Expression of endothelial nitric oxide synthase in developing rat kidney

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Han, Ki-Hwan, Jung-Mi Lim, Wan-Young Kim, Hyang Kim, Kirsten M. Madsen, and Jin Kim. Expression of endothelial nitric oxide synthase in developing rat kidney. Am J Physiol Renal Physiol 288: F694–F702, 2005. First published November 30, 2004; doi:10.1152/ajprenal.00085.2004.—Endothelium-derived nitric oxide (NO) is synthesized within the developing kidney and may play a crucial role in the regulation of renal hemodynamics. The purpose of this study was to establish the expression and intrarenal localization of the NO-synthesizing enzyme endothelial NO synthase (eNOS) during kidney development. Rat kidneys from E14, E16, E18, and 20-day-old (E20) fetuses and 1 (P1), 3 (P3), 7 (P7), 14 (P14), and 21-day-old (P21) pups were processed for immunocytochemical and immunoblot analysis. In fetal kidneys, expression of eNOS was first observed in the endothelial cells of the undifferentiated intrarenal capillary network at E14. At E16, strong eNOS immunoreactivity was observed in the endothelial cells of renal vesicles, S-shaped bodies (stage II glomeruli), and stage III glomeruli at the corticomedullary junction. At E18–20, early-stage developing glomeruli located in the subcapsular region showed less strong eNOS immunoreactivity than those of E16. The eNOS-positive immature glomeruli were observed in the nephrogenic zone until 7 days after birth. In fetal kidneys, eNOS was also expressed in the medulla in the endothelial cells of the capillaries surrounding medullary collecting ducts. After birth, eNOS immunostaining gradually increased in the developing vascular bundles and peritubular capillaries in the medulla and was highest at P21. Surprisingly, eNOS was also expressed in proximal tubules, in the endocytic vacuolar apparatus, only at P1. The strong expression of eNOS in the early stages of developing glomeruli and vasculature suggests that eNOS may play a role in regulating renal hemodynamics of the immature kidney.

NITRIC OXIDE (NO) IS A LIPOPHILIC gas with unique physiologic properties and plays an important role in the regulation of renal blood flow (RBF) and glomerular filtration rate (GFR) in the kidney (3, 6, 15, 19, 21, 22). The production of NO is mediated by the three different isoforms of NO synthase (NOS): 1) neuronal (nNOS, NOSI), 2) inducible (iNOS, NOSII), and 3) endothelial (eNOS, NOS III). Expression of the three major NOS isoforms is cell type specific and subject to distinct control mechanisms (16). In the adult kidney, nNOS is predominantly expressed in tubular epithelial cells of the macula densa and inner medullary collecting duct (2, 18). Tojo et al. (30) identified expression of iNOS protein in the thick ascending limb of normal rats with two different iNOS antibodies and expression in the proximal tubule and intercalated cells of the collecting duct with a polyclonal anti-iNOS antibody. eNOS has been localized in the endothelium of the intrarenal arteries, the glomerular capillaries, the afferent and efferent arterioles, and the medullary vasa recta (2, 30).

Although the role of NO in the regulation of renal hemodynamics has been well established in the adult, recent studies point to perhaps an even more critical role for NO in maintaining basal RBF and GFR in the developing kidney. During development, the immature kidney exhibits low RBF and high renal vascular resistance (RVR) (1, 9, 13). These hemodynamic conditions are maintained by highly activated vasoconstrictors such as the renin-angiotensin system and contribute to the newborn’s low GFR, a major factor in neonatal pathophysiology. In fact, renal renin expression peaks perinatally, at a time where it is found in smooth muscle cells all along the renal resistance vessels (7, 8, 17). Functioning as a vasodilator, NO might counterbalance the activated vasoconstrictors in the fetal and postnatal maturing kidney (24, 25, 28).

This greater functional role for NO in the developing kidney might be due to a different expression of NOS compared with adult expression. Studies by Solhaug et al. (26, 27) demonstrated that the highest expression of nNOS occurred immediately at birth in the pig, especially in the renal medulla, and progressively declined with age to the lowest levels in the adult. Fischer et al. (5) investigated the postnatal development of both nNOS and renin signals and analyzed the age-dependent intrarenal distributions in the rat. Although there was parallelism between the intrarenal distribution of nNOS and renin signals, the intensity of nNOS and renin expression along the developmental time axis was not parallel. The abundance of nNOS reached its maximum on postnatal day 6 (P6), whereas renin expression was highest on day 2 (5). At birth, eNOS also showed critical expression in the newborn pig kidney but rapidly decreased after birth, reaching its lowest point at 7 days of age and returning by 14 days to adult levels (27). Moreover, cortical eNOS was proportionately greater than medullary eNOS in the immature kidney, whereas medullary eNOS was greater than cortical in the adult (27). These findings suggest that eNOS may be a critical participant in regulating renal cortical hemodynamics and GFR during early renal maturation. Also, studies by Sener and Smith (21, 22) measured RBF and GFR in the developing sheep kidney and...
21-day-old (P21) pups. Kidneys from adult male rats (E18-E14-E16) -, 16 (P1) -, 18 (P3) -, and 20-day-old (P14) fetuses and 1 (P1) -, 3 (P3) -, 7 (P7) -, 14 (P14) -, and 21-day-old (P21) pups. Kidneys from adult male rats (n = 3) served as a positive reference for the immunocytochemical studies. The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The kidneys were preserved by in vivo perfusion through the heart or abdominal aorta. The animals were initially perfused briefly with PBS (298 mosmol/kgH2O, pH 7.4) to rinse away the blood. For Western blot analysis, the right kidneys of animals were excised after the renal artery was clamped. For immunocytochemical studies, the left kidneys were then perfused with a periodate-lysine-2% paraformaldehyde (PLP) solution for 5 min. After perfusion, the kidneys were removed and cut into 1- to 2-mm-thick slices that were fixed additionally by immersion in the PLP solution overnight at 4°C. Sections of tissue were cut transversely through the entire kidney on a vibratome at a thickness of 50 µm and processed for immunocytochemical studies using a horseradish peroxidase preembedding technique.

Antibodies. The antibodies used were purified mouse IgG raised against the COOH terminus of human eNOS (catalogue no. N30020, Transduction Labs, Lexington, KY).

Light and electron microscopic immunocytochemistry. Fifty-micrometer vibratome sections were processed for immunocytochemistry using an indirect preembedding immunoperoxidase method, as previously described (11, 12). All sections were washed with 50 mM NH4Cl in PBS three times for 15 min. Before incubation with the primary antibody, the sections were pretreated with a graded series of ethanol followed by incubation for 3 h with PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin (solution A). The tissue sections were then incubated overnight at 4°C with the antibody against eNOS (1:1,000) diluted in 1% BSA-PBS (solution B). Control incubations were performed in solution B without the primary antibody. After several washes with solution A, the sections were incubated for 2 h in a peroxidase-conjugated donkey anti-mouse IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in solution B. The tissues were rinsed first in solution A and subsequently in 0.05 M Tris buffer (pH 7.6). For the detection of horseradish peroxidase, sections were incubated in 0.1% 3,3′-diaminobenzidine in 0.05 M Tris buffer for 5 min, after which H2O2 was added to a final concentration of 0.01% and the incubation was continued for 10 min. After being washed with 0.05 M Tris buffer, the sections were dehydrated in a graded series of ethanol. From all animals, 50-µm-thick vibratome sections through the entire kidney were embedded in poly/Bed-812 resin (Polysciences, Warrington, CA) between polyethylene vinyl sheets and examined by light microscopy. Sections from the cortex of PI kidney were also excised and glued onto empty blocks of poly/Bed-812 resin. After examination of 1-µm semithin sections by light microscopy, ultrathin sections were cut, stained with lead citrate, and photographed with a transmission electron microscope (JEOL 1200EX, Tokyo, Japan).

Western blot analysis. For Western blot analysis, kidneys were perfused through the heart or abdominal aorta with cold PBS to rinse out the blood, and protein was extracted, as previously described (11). Kidneys were homogenized in lysis buffer containing 20 mM Tris-HCl, 1% Triton X-100, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide, 1 mM EDTA, 10 µM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 3,000 g for 20 min at 4°C. After determination of protein concentration in the supernatant by the Coomassie method (Pierce, Rockford, IL), samples were loaded (30 µg/lane) and underwent electrophoresis on sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. Proteins were transferred to nitrocellulose membranes by electroblootting. To reduce nonspecific antibody binding, the membranes were blocked with 5% nonfat dry milk for 30 min at room temperature and then incubated for 24 h at 4°C with affinity-purified anti-eNOS (1:1,000). After three washes, the blot was incubated with peroxidase-conjugated donkey anti-mouse IgG (1:1,000) for 2 h at room temperature.
Samples were visualized using an enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, UK) after a 5- to 30-min exposure at room temperature. Human endothelial lysate derived from an aortic endothelium cell line was used as a positive control. Negative controls were performed without the primary antibody, and on BSA that does not contain eNOS. Densitometric analysis was performed using Zero-Dscan software in the Eagle EYETMII Still Video System (Stratagene, La Jolla, CA). Results are presented as means ± SD, and all statistical analyses were performed with SigmaPlot software for Windows, version 8.0. Comparisons between groups were made using an unpaired t-test. P values < 0.05 were considered significant.

RESULTS

Localization of eNOS in adult rat kidney. eNOS was expressed in the endothelial cells of almost all blood vessels in the adult kidney except the venous system (Figs. 1 and 2). eNOS immunoreactivity was observed in the endothelial cells of glomeruli and peritubular capillaries in the cortex (Fig. 2, A and B) and in the endothelial cells of vascular bundles in the medulla (Fig. 2, E and F). However, the staining intensity was much stronger in the endothelial cells of the renal medulla than in those of the renal cortex. Considerable eNOS immunostaining was also detected in the endothelium of arcuate and interlobular arteries (Fig. 2, C and D). The uriniferous tubules including proximal tubules and macula densa showed no eNOS immunoreactivity in the adult rat kidney.

Expression of eNOS in fetal kidneys. At E14, eNOS immunoreactivity was observed in both the cortex and medulla, in the endothelial cells of capillary networks surrounding developing nephrons and the ureteric bud. The eNOS-positive capillary networks within the developing kidney were connected to the small blood vessels branched from the dorsal aorta at this age (Fig. 3A). At E16–20, eNOS immunostaining was observed in glomeruli of various developmental stages in the cortex and in the capillaries surrounding collecting ducts in the medulla (Fig. 3, B and C, E20: not shown). In the cortex, eNOS

Fig. 2. Light micrographs of 50-μm-thick vibratome sections from an adult rat kidney illustrating eNOS immunostaining. A–D: higher magnification of renal cortex. eNOS immunoreactivity was observed in the endothelial cells (arrows) of the glomerulus (A), peritubular capillary network (B), arcuate artery (C), and interlobular artery (D). There was no eNOS immunoreactivity in the vascular smooth muscle cells (arrowheads) and in the macula densa (A, inset). E and F: higher magnification of outer medulla and IM, respectively. The eNOS-positive endothelial cells were more abundant in the outer medulla and showed more intense labeling (double arrows) in the IM. Magnifications: ×432.
was strongly expressed in the endothelium of capillaries surrounding vesicles (stage I nephron) and in the cleft of S-shaped bodies (stage II nephron) mainly located at the corticomedullary junction at E16 (Fig. 3D). At E18–20, the eNOS-positive vesicles and S-shaped bodies appeared at more cortical (subcapsular) regions and looked smaller than those of E16. The immunostaining of eNOS was also observed in more mature glomeruli of stage III nephrons at E18–20 (Fig. 3, F and G, E20: not shown).

In fetal kidneys, eNOS was expressed not only in the arterial system but also in the venous system, including the arcuate vein and its branches (Fig. 3, E and G). However, the intensity of staining was much greater in the arterial system. There was no eNOS immunoreactivity in the developing uriniferous tubule, including the proximal tubules.

Expression of eNOS in postnatal kidneys. During development after birth, there were two major changes in the expression of eNOS in the kidney. In the cortex, eNOS immunoreactivity was strongest at P1 and gradually decreased after birth. In contrast, immunostaining gradually increased in the medulla after birth and became strongest at P21 (Fig. 4). There was no observable immunostaining in control incubations without the primary antibody (Fig. 5). At P1, eNOS was expressed in the developing glomeruli of various stages, including vesicles and S-shaped bodies, and in the peritubular capillaries in the cortex. Immature glomeruli with eNOS immunoreactivity were observed until P7 in the cortex. At P14–21, almost all glomeruli had matured to the adult form, and moderate eNOS immunoreactivity remained in the endothelial cells of glomeruli and peritubular capillaries through to the adult stage (Fig. 6, A–D). Although the labeling of postnatal glomerular eNOS appeared less intense than those of fetal glomerular eNOS, in general the endothelial cells showed still greater eNOS immunoreactivity in the cortex than in the medulla at P1 (Fig. 6, A and E) and P3 (Fig. 7). Strikingly, there was also strong labeling of eNOS in the proximal tubules at P1 (Fig. 4A and 6A).
scopy revealed that eNOS immunostaining was located primarily in vacuoles and lysosome-like structures in the proximal tubules (Fig. 8). The immunoreactivity in the proximal tubules was observed only at P1 and disappeared from P3.

In the medulla, expression of eNOS was observed in developing vascular bundles and peritubular capillaries, which gradually increased in number and were fully developed at P21 (Fig. 4). Interestingly, the P7 medulla appeared to have less eNOS staining than those of the other ages (Fig. 6, E–G). Expression of eNOS remained in the endothelial cells of the arterial system, including the arcuate and interlobular arteries, but gradually disappeared from the venous system after birth (not shown).

Western blot analysis. Expression of eNOS proteins was determined in fetal and postnatal kidneys. Immunoblot evaluation of whole kidney protein showed a specific band at 135 kDa. Determination of the relative abundance by densitometry demonstrated an abrupt increase in whole kidney eNOS from E20 to the newborn stage. Whole kidney eNOS significantly decreased to a low level at P7 and then rebounded to highest levels at P14 and P21 (Fig. 9). The level of eNOS expression increased further from P21 to the adult stage (Fig. 10).

Because eNOS expression showed distinct patterns in the cortex and medulla after birth, we determined cortical and medullary eNOS protein content in P1, P7, and P21 pups and in adult rats. After birth, eNOS abundance gradually decreased in the cortex, whereas it increased in the medulla. Although a dip in eNOS abundance was observed in the medulla at P7, this was not statistically significant (Fig. 11).

DISCUSSION

The present study provides the first morphological description of eNOS expression in the developing rat kidney. The eNOS immunostaining was first observed in the capillary network in the kidney at E14. During renal maturation, the expression of eNOS in the immature kidney showed a unique corticomedullary pattern distinct from that observed in the adult. In the fetuses and newborn animals, the intensity of eNOS immunostaining was much stronger in the developing renal vascular system in the renal cortex than in the medulla. As the kidney matured after birth, the strong eNOS immunostaining gradually decreased in the cortex. In contrast, in the renal medulla, the expression of eNOS gradually increased after birth in the endothelial cells of the vascular bundles and capillaries and became highest in the adult kidney. Furthermore, we demonstrated a significant dip in whole kidney eNOS expression in the postnatal period at P7 by Western blot analysis, which was also consistent with the results of immunocytochemistry. These findings are in general agreement with the previous descriptions and confirmatory of previous demonstration of postnatal renal eNOS expression.

The functional significance of the strong expression of eNOS in the cortex during renal maturation is not certain.
However, it is likely that eNOS is involved in the regulation of renal hemodynamics including GFR in the immature kidney. As an endothelium-derived relaxing factor, NO plays a major role in maintaining basal renal vascular tone in the adult kidney (6, 15, 19). Because the developing kidney exhibits high RVR and low RBF, which contribute to the newborn’s low GFR, NO has been shown to be an important regulator of renal hemodynamics in the developing kidney (14, 21, 22, 25, 29). Highly activated vasoconstrictor mechanisms such as the renin-angiotensin system are believed to maintain the high RVR during renal maturation (7, 8, 10, 17, 20), and recent studies have demonstrated critical hemodynamic interactions between NO and the renin-angiotensin system in the developing kidney (24, 28).

It was surprising that eNOS immunostaining was also observed in proximal tubules at P1. Electron microscopy revealed that eNOS immunostaining was mainly located in the endocytic vacuolar apparatus, indicating that it was reabsorbed from the glomerular filtrate. The transient expression of eNOS in proximal tubules only at P1 implies a critical change in cortical renal hemodynamics immediately after birth. Interestingly, eNOS-positive proximal tubules appeared over the juxtamedullary and midcortical regions rather than in the subcapsular regions (Fig. 4A). This pattern may be associated with a critical expression of renin in the newborn kidney. Fischer et al. (5) reported that both renin and renin mRNA occupied extended regions of the afferent glomerular arterioles of juxtamedullary and midcortical nephrons, when renin was highest on P2 in the rat kidney. However, the physiological importance remains to be established.

Another putative role of eNOS in the developing kidney may involve the regulation of growth and/or branching of the vascular tree during renal maturation. There is substantial evidence that endothelium-derived NO is a major mediator of angiogenesis (4). We have demonstrated that the expression of eNOS was already notable at E14 when the vascular system

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**Fig. 6.** Light micrographs of 50-μm-thick vibratome sections from renal cortex (A–D) and medulla (E–G) of 1 (A and E), 3 (B), 7 (C and F), 14 (D), and 21 (G)–day-old rat pups illustrating immunostaining for eNOS. eNOS immunoreactivity in the cleft (arrows) of immature glomeruli (iG) became less intense in the cortex after birth, whereas the staining intensity in the vascular endothelial cells (arrows) in the medulla was increased after birth. Note intense eNOS labeling in the proximal tubule (PT), only at P1. Magnification: ×330 (A–D); ×480 (E–G).
existed as an undifferentiated capillary plexus in the kidney. In fetal kidneys, eNOS immunostaining was detected not only in the arterial system but also in the developing venous system, including the arcuate vein and its branches (Fig. 3). At E16–P7, the expression of eNOS was remarkably strong in the immature glomerular capillaries in the nephrogenic zone. These observations imply that eNOS may play a role during the development and differentiation of the renal vasculature. However, studies using a knockout mouse model lacking the eNOS gene revealed apparently normal renal vascular and glomerular anatomic formation (23).

In this study, we demonstrated that eNOS was strongly expressed in the immature vascular system and glomeruli in the developing rat kidney. Interestingly, the expression of eNOS was observed in endosome- or lysosome-like structures (arrows) in the cytoplasm (Fig. 8).
eNOS exhibited a distinct corticomedullary pattern during kidney development. These results suggest that eNOS may be differently regulated in the two regions and play a role in the regulation of renal hemodynamics during renal maturation.

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