Expression of endothelial nitric oxide synthase in the postnatal developing porcine kidney

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Solhaug, Michael J., Usa Kullaprawithaya, Xui Q. Dong, and Ke-Wen Dong. Expression of endothelial nitric oxide synthase (eNOS) is unknown. The purpose of this study was to characterize eNOS expression during maturation and compare this to neuronal NOS (nNOS). The experiments measured whole kidney eNOS mRNA expression by RT-PCR and protein content by Western blot, as well as cortical and medullary protein content in piglets at selected postnatal ages and in adult pigs. Whole kidney eNOS mRNA was compared with nNOS. Whole kidney eNOS expression decreased from the newborn to its lowest at 7 days, returning by 14 days to adult levels. This eNOS mRNA pattern contrasted with nNOS, which was highest at birth, and progressively decreased to its lowest level in the adult. At birth, cortical eNOS protein was greater than medullary, contrasting with the adult pattern of equivalent levels. In conclusion eNOS is developmentally regulated during early renal maturation and may critically participate in renal function during this period. The eNOS developmental pattern differs from nNOS, suggesting that these isoforms may have different regulatory factors and functional contributions in the postnatal kidney.

NO, functioning as an efficient intracellular messenger, is produced from the single amino acid precursor L-arginine and is mediated by NOS. In the kidney the three isoforms of NOS: 1) neuronal (nNOS, NOS 1), 2) inducible (iNOS, NOS 2), and 3) endothelial (eNOS, NOS 3) demonstrate specific localization within the nephron (1, 12). The nNOS isoform is predominantly detected in tubular epithelial cells of the macula densa and inner medullary collecting duct. This isoform has been characterized in the adult in several species (13) and in developing kidney in the rat (7) and pig (22). After birth nNOS mRNA gene expression and protein follow a parallel, distinct pattern (22). The highest expression of whole kidney nNOS occurs immediately at birth. Thereafter, nNOS expression progressively declines with age to the lowest levels in the adult (22). Furthermore, nNOS undergoes differential corticomedullary regulation. Medullary nNOS is proportionately greater than cortical in the immature kidney than the adult (22). Given the unique developmental pattern of nNOS, this isoform may contribute to the functional significance of NO throughout the entire period of renal maturation.

However, other NOS isoforms may contribute to neonatal renal hemodynamics as well, in particular eNOS. This isoform has been localized to the renal vasculature in the adult (12), including the glomerular afferent and efferent resistance arterioles and vasa recta. eNOS is also found in tubular structures: proximal tubule, medullary thick ascending limb, and collecting ducts (12). Furthermore, eNOS expression and activity may be greater in the medulla than the cortex in the adult rat (24). However, the expression of eNOS during postnatal renal maturation has not been studied.

The purpose of these experiments therefore was to characterize eNOS expression throughout the entire period of postnatal renal maturation and compare this pattern to that of nNOS. The experiments quantified whole kidney eNOS mRNA expression by RT-PCR and eNOS protein content by Western blot in piglets (newborn, 3, 7, 14, and 21 days old) and in adult pigs. Cortical and medullary eNOS protein was quantified.
in newborn and 7-day-old piglets and in adult pigs. Additionally, to compare the developmental patterns of eNOS and nNOS in the same tissue specimen, the relative abundance of eNOS and nNOS mRNA by RT-PCR was measured in piglets (newborn and 7 days old) and in adult pigs.

MATERIALS AND METHODS

Animals. All experiments were performed on mixed breed swine obtained from the same local vendor. All animals were transported and housed under the same conditions. Piglets were procured as littermate pairs. Kidneys of preweanling piglets (2 h, 3, 7, 10, and 14 days old) were harvested on arrival. Kidneys of weaned piglets (21 days old) and adults >70 days of age were harvested 24 h after arrival to allow for dietary and climatic adjustment.

Tissue preparation. After death with a euthanizing solution, 390 and 50 mg/ml phenothion, kidneys are immediately removed from the animals (piglets and adult pigs) and coronally sectioned. The coronal sections are cut into segments that conserve the corticomedullary architecture from the lower and upper pole and mid-kidney regions for whole kidney analysis. Cortex and medulla were carefully separated under a dissecting microscope. The prepared tissue is then immediately frozen in liquid nitrogen and stored at −80°C for future use in experiments.

Western blot analysis. Western blots were performed on whole kidney homogenates, cortex, and medulla from tissue processed as previously described. Whole kidney Western blots were performed on piglets (newborn, 3, 7, 14, and 21 days old) and adult pigs. These age groups were selected for this study to provide a representative analysis of the postnatal developmental spectrum. Separate experiments involved Western blots on the cortex and medulla in piglets (newborn and 7 days old) and adult pigs. These ages were chosen based on the data obtained from the larger age group sampling. The protein concentration of the homogenates was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). Ten micrograms of the total protein was loaded and separated on a 6% denaturing SDS-PAGE. The proteins were then blotted onto nitrocellulose (Hy-Bond, Amersham, Arlington Heights, IL) by dry electroblot for 1 h. The blots were allowed to air dry for 1 h and then blocked overnight at 4°C with 6% nonfat dry milk in Tris-saline buffer. Because antiporcine eNOS is not commercially available, we used a human eNOS antibody. Selection of this antibody is based on 90% homology of the human protein to the porcine eNOS protein. The difference between the human and porcine eNOS protein is only a total of 15 amino acids. The human eNOS antibody used for this Western blot analysis was purchased from Alexis Biochemicals (San Diego, CA), who provided the amino acid analysis. The blots were then washed twice in Tris-saline buffer and then incubated with the human eNOS isoform-specific antibody at a dilution of 1:4,000 in Tris-saline buffer for 2 h at room temperature. The blots were washed four times in Tris-saline buffer and incubated with horseradish peroxidase-conjugated rabbit immunoglobulin (Amersham, Piscataway, NJ) antibody for 1 h at room temperature. Finally, the blots were washed four additional times in Tris-saline buffer, and the specific proteins were detected by enhanced chemiluminescence (Amersham). Pulmonary artery from newborn animals was used as a positive control. Negative controls were performed as described previously without the primary antibody, and on recombinant human Zp3, a zona pellucida protein that does not contain eNOS.

Isolation of total RNA. The method for isolation of RNA is described by Cathala et al. (5). Briefly, frozen tissues obtained by methods described previously (~0.5 g) are crushed before being transferred into centrifuge tubes containing 4 ml of lysis buffer [5 M guanidine monothiocyanate, 10 mM EDTA, 50 mM Tris·HCl, pH 7.5, and 8% (vol/vol) α-mercaptoethanol added just before use]. The tissues are homogenized and then centrifuged at 2,000 g at 4°C for 10 min. Seven volumes of 4 M LiCl is added to the supernatants, mixed well, and left at 4°C overnight. The mixture is pelleted by centrifugation at 11,000 g, 4°C for 90 min, resuspended in 7 ml of 3 M LiCl, and centrifuged at 11,000 g, 4°C for an additional 60 min. The pellets are resuspended in 4 ml of cold solubilization buffer (0.1% SDS, 1 mM EDTA, and 10 mM Tris·HCl, pH 7.5). A 0.05 volume of 7.5 M ammonium acetate is added to RNAs samples and then extracted with one volume of phenol/chloroform (1:1). One volume of cold 2-propanol is added to the RNA sample and kept at −20°C overnight. After centrifugation, the RNA sample is air dried and resuspended in diethyl pyrocarbonate water. The concentration of RNA is measured using spectrophotometer at 260 and 280 nm optical density. Integrity of RNA is determined by agarose gel electrophoresis.

Quantitative RT-PCR. Quantitative RT-PCR was performed on whole kidney homogenates from tissue processed as described previously. Separate experiments were performed on samples from piglets (newborn, 3, 7, 14, and 21 days old) and adult pigs. These age groups were selected for this study to provide a representative analysis of the postnatal developmental spectrum. Quantitative RT-PCR was also performed on whole kidney samples obtained from the same animal in piglets (newborn and 7 days old) and adult pigs. These ages were chosen based on the data obtained from the larger age group sampling. A pair of primers specifically complementary to porcine eNOS cDNA is employed. The sense strand primer is located between base +1818 and +1839 (5'-AGGAGGTGACAAGCGCA TAC-3') and antisense is located between base +2236 and +2256 (5'-AAGAT GAGTGAGAGAGA TAC-3'). Meanwhile, sense strand primer of nNOS is base at +1585 to +1606 (5'- TCAAGTCTCAAGAC TGGGAG-3', according to human nNOS sequence) and antisense is located at base +1940 to +1960 (5'-CCTGACGTCT CGACCTGTGG-3'). Ten micrograms of total RNA is hybridized with oligo(dT) in RT buffer (BRL), and the reaction carried out with Moloney-Murine Leukemia Virus reverse transcriptase (BRL) for 2 h at 37°C. The reaction terminates by heating for 15 min at 68°C and is diluted to a final volume of 100 μl. Ten microliters of the reverse transcription reaction are added to a final volume of 50 μl in 10 mM Tris·HCl, pH 8.4; 2.5 mM MgCl2, 250 μM dATP, dGTP, dCTP, and TTP; 0.5 μg each of sense and antisense strand primers, and 2.5 U Taq polymerase (Perkin Elmer/Cetus, Norwalk, CT). The polymerase amplification is carried out for 30 cycles (30 s) and a 72°C denaturing cycle (1 min), a 55°C annealing cycle (33 s), and 72°C extension cycle (2 min), followed by a final extension at 72°C for 10 min. The PCR products are then visualized by electrophoresis in an agarose gel with ethidium bromide. RNA sample from newborn piglet without RT was used as a negative control. [35S]dCTP is included for the RT-PCR quantitation studies. [35S]dCTP (1 μM) is used for each reaction. For the standard curve, eNOS cDNA purified from gel, is quantitated by measuring the absorbance at 260 nm and used as DNA template for quantitation. The RT-PCR products are separated on a 4% polyacrylamide gel, and radioactivity is quantitated by a Molecular Dynamic phosphorImager (Image Quant Software Program). The level of light emission (proportional to radioactivity) is quantitated by Molecular Dynamic phosphorImager (Image Quant Software Program).
activity) is plotted against the known amount of standard DNA to generate a regression line from which the content of the specific PCR product in each sample is computed. In parallel experiments, the same amount of different porcine kidney samples is performed by RT-PCR with a pair of human cyclophilin primers as an internal control.

Statistical analysis. For all age groups, experiments were performed on kidney samples obtained from five separate animals. All assays were repeated at least three times. Results are expressed as means ± SE, with statistical significance at \( P < 0.05 \). The data were evaluated by analysis of variance. Comparisons among means were performed using Newman-Keuls test.

RESULTS

Whole kidney eNOS mRNA levels by quantitative RT-PCR. Whole kidney eNOS RT-PCR was determined in piglets (newborn, 3, 7, 14, and 21 days old) and adult pigs. RT-PCR results in a single amplification band at 439 bp only from the tested samples but not from the RNA without RT (negative control) as shown in Fig. 1. To quantify the eNOS mRNA level by RT-PCR, a standard curve was generated using eNOS cDNA as a DNA template. To determine the optimum amount of DNA template for PCR as well as the optimum PCR cycle, a series of PCR experiments with different concentration of eNOS cDNA and different PCR cycles were performed. As shown in Fig. 1, when 0.125–8 pg of DNA template and 35 PCR cycles were used, the linear relationship between the concentration and incorporation with \([\text{35S}]\text{dCTP} \) was obtained. The expression of eNOS mRNA is higher in the newborn kidney and significantly decreases to the lowest level at day 7. After this decrease, the expression level of eNOS mRNA significantly increases in the developing porcine kidney by day 14. The eNOS mRNA expression remains at this level throughout maturation to the adult.

Whole kidney eNOS protein content by Western blot. Western blot analysis was performed throughout the renal maturational spectrum to determine whether the protein content of eNOS in the developing porcine kidney has the same pattern as mRNA expression. As shown in Fig. 2, Western blot analysis of samples of whole kidney homogenates demonstrated that eNOS protein content has a pattern similar to that of mRNA gene expression. The higher protein content is first observed in newborn and then significantly decreases to the lowest level at day 7. The eNOS protein content increases to day 14 and remains at this level through to the adult.

Cortical and medullary eNOS protein content by Western blot. Based on the observation that whole kidney eNOS dropped to the lowest level at 7 days of age, eNOS protein content was measured in the cortex and medulla in animals of selected ages. Therefore, cortical and medullary eNOS protein content was determined in piglets (newborn and 7 days old) and adult pigs. The pattern of changes in eNOS protein content in cortex and medulla during postnatal renal development are demonstrated in Fig. 3. Cortical eNOS is greater than medullary at birth and 7 days of age, compared with the adult, which shows the reverse pattern, with medullary relatively equivalent to cortical. The newborn demonstrated the greatest amount of cortical eNOS. Medullary eNOS was greatest in the adult and least in the 7-day-old piglet.

Comparison of whole kidney mRNA levels between nNOS and eNOS by RT-PCR. Our previous description of the postnatal renal developmental pattern of nNOS (22) and the results from the studies of eNOS expression were obtained from experiments performed in separate kidney samples. To more carefully study the relative abundance of eNOS and nNOS mRNA expression during postnatal renal maturation, experiments

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**Fig. 1.** Quantification of the endothelial nitric oxide synthase (eNOS) mRNA gene expression level by RT-PCR in piglets (newborn, and 3, 7, 14, 21 days old) and adult pigs. A: autoradiograph of polyacrylamide gel of \([\text{35S}]\text{PCR} \) amplification of eNOS cDNA. B: standard curve generated from radioactivity present in RT-PCR product plotted against varying quantities of eNOS cDNA. C: autoradiograph of polyacrylamide gel of \([\text{35S}]\text{RT-PCR} \) product amplified from the first strand cDNA of porcine kidney in different ages. D: level of eNOS mRNA during the period of renal maturation, expressed relative to 1 pg of eNOS cDNA. Assay was repeated 3 times. Results are expressed as means ± SE; \( n = 5 \) for each age. \( ^*P < 0.05, \) 7 days vs. all ages.
were performed on samples obtained from the same kidney. RT-PCR analysis of eNOS and nNOS mRNA gene expression therefore was performed on newborn and 7-day-old piglets and adult pigs. cDNA from eNOS and nNOS were used as positive control to distinguish the PCR product of eNOS and nNOS. As shown in Fig. 4, in the newborn kidney, both eNOS and nNOS are expressed at a high level. However, at day 7 mRNA level of eNOS dramatically decreases, whereas mRNA level of nNOS only slightly decreases. As described in our previous studies on nNOS expression during renal maturation, (22) the mRNA level of nNOS decreases to...
the lowest level in the adult kidney. In contrast, mRNA levels of eNOS in the adult returns to the same high level as in the newborn kidney.

DISCUSSION

These studies are the first to describe eNOS expression in the postnatal developing kidney. Both whole kidney eNOS mRNA and protein content demonstrate parallel changes throughout the period of renal maturation. From a high level at birth, the expression of this isoform rapidly decreases to its lowest point at 7 days of age. Thereafter, eNOS abundance increases to approximate the newborn and is sustained at this level to the adult. The developmental pattern of eNOS contrasts with that of nNOS. Experiments in this study also demonstrate the mRNA expression of the two isoforms in the same kidney sample. In contrast to the fluctuating eNOS levels that return to adult equivalent levels during maturation, nNOS remains greater than the adult throughout renal development. The findings in this series of experiments confirm our previous description of nNOS mRNA and protein expression in the postnatal kidney performed in separate samples at ages representative of the entire developmental spectrum (22). Given the developmental differences between whole kidney eNOS and nNOS, the timing of their contribution to NO function during renal maturation may differ as well. Whereas nNOS may undergo regulation and may participate in renal function throughout the period of renal development, the most important impact of eNOS may occur in a narrower postnatal window, in the first days of the maturing kidney.

Critical changes occur in renal function after birth. Compared with the adult, the newborn kidney has low RBF and low GFR because of the high renal vascular resistance (RVR) produced by highly activated vasoconstrictors. Synchronous increases in RBF and GFR ensue during development, mediated by decreases in RVR in the intrarenal resistance vessels (21). The most abrupt hemodynamic changes occur immediately after birth, with a rapid rise in RBF and GFR. In the newborn kidney, although overall RBF is low, cortical blood flow is proportionately greater than in the adult. As RBF abruptly increases in the early neonatal period, most of the increase occurs in the cortex. Thereafter, continued maturation of renal hemodynamics proceeds progressively with age to adult capability. Compared with the adult, NO has a greater role in immature renal function (21). Functioning as a vasodilator, NO counterbalances highly activated vasoconstrictor mechanisms, such as the renin angiotensin system (20, 23). In the adult kidney, eNOS is strategically located in afferent and efferent arterioles (1, 12, 25) to modulate cortical renal hemodynamics. Thus increased cortical amounts of eNOS at birth would further support the timing of eNOS participation at this time of development.

Indeed, our studies show that eNOS undergoes differential changes in cortical and medullary expression during renal development. In the newborn and 7-day-old kidney, cortical eNOS protein content is greater than medullary, a pattern that is different in the adult with cortical and medullary eNOS expression almost the same. Also, the newborn demonstrates the largest abundance of cortical eNOS, whereas the adult has relatively equivalent amounts of cortical and medullary eNOS protein. The changing corticomedullary patterns of eNOS in the immature kidney are of particular interest in light of recent descriptions of medullary eNOS in the adult rat under basal and stimulated conditions and our previous studies of nNOS corticomedullary changes during development. NOS enzymatic activity, without distinguishing between the individual isoforms in normal adult rats, is greatest in the medulla (27). With specific regard to medullary eNOS, in the normal adult rat, Mattson and Higgins (15) reported increased medullary expression of this isoform. Wu et al. (27) demonstrated increased levels of eNOS mRNA in not only the vasa recta, but also in the inner medullary collecting duct, both of which were greater than cortical eNOS. Although cortical and medullary eNOS was approximately the same under basal conditions in untreated rats, deoxycortisone ace-
tate treatment of normal rats increased both cortical and medullary eNOS, with most of the medullary increase occurring in tubular structures (16). Furthermore, the corticomedullary pattern of eNOS during renal development also differs from nNOS. We have reported previously that at all ages studied, newborn, 14-day-old piglets, and adult pigs, nNOS medullary expression was greater than cortical. The medullary expression was proportionately the greatest in the newborn, at the time that whole kidney nNOS is the most abundant (22). The cortical predominance of eNOS in the newborn and during the first days after birth supports the possibility that eNOS is a critical participant in cortical renal hemodynamics immediately after birth.

Both whole kidney and corticomedullary eNOS expression undergo developmental regulation during postnatal renal maturation. However, this developmental pattern of eNOS differed from nNOS. This observation is consistent with our previous descriptions of nNOS in postnatal renal development (22). These differences between the two NOS isoforms suggest that they are regulated either by different factors or by different mechanisms. Potential factors relevant to the regulation of eNOS in the developing kidney include cell growth and proliferation, hormones, such as estrogen, growth factors, and vasoconstrictors, such as angiotensin II. The state of cell growth influences eNOS expression; however, the direction of eNOS change remains disputed. Most studies report an up-regulation of eNOS mRNA in proliferating cells associated with no change in transcription rates but a posttranscriptional stabilization of mRNA (8, 18). Immediately after birth the newborn has high levels of estrogen and estrogen receptors (10). Estrogen upregulates eNOS mRNA and protein expression in a variety of experimental situations (8), perhaps from increased gene transcription (26). Two growth factors appear to regulate eNOS. Transforming growth factor-β1, which is highly expressed during renal development (2, 6) upregulates eNOS when applied directly to cell cultures or when stimulated by high salt diet (8, 28). Also, vascular endothelial growth factor (VEGF)-A, a participant in the formation of glomerular vessels, upregulates eNOS in cell culture (3, 14) through the VEGF receptor-2 (14). Angiotensin II regulates eNOS in adult rat renal resistance arterioles, perhaps via the AT-1 receptor subtype (24). To further understand the role of eNOS in the immature kidney, studies are needed to localize eNOS in the immature kidney, quantify it in selective nephron components in the cortex and medulla, and examine mechanisms producing its unique regulatory pattern.

In summary, eNOS mRNA and protein expression demonstrate distinct parallel changes during the early period of postnatal renal maturation, dipping from newborn levels to a low at 7 days and then returning to newborn and adult equivalent abundance. This contrasts to the developmental pattern of nNOS, which is greatest at birth, then progressively declines though the period of renal maturation to the lowest levels in the adult. At birth and in the early days of development, cortical expression is greater than medullary, compared with the adult, in which cortical and medullary eNOS are relatively the same.

In conclusion, eNOS undergoes both whole kidney and corticomedullary developmental regulation in the early postnatal renal maturation and may critically participate in renal hemodynamics of the immature kidney during this period of development. The contrasting patterns between eNOS and nNOS suggest that these isoforms may be regulated by different mechanisms and may contribute to NO function differently during renal maturation.

**Perspectives**

NO functions as a vital vasodilator in the immature kidney, functioning to counterbalance highly activated vasoconstrictor systems to maintain RBF and GFR. However, how and when NO participates in maturing renal hemodynamics is not well understood. The discovery that eNOS renal expression undergoes developmental changes in the first few days after birth provides a clue into the role of NO immediately after birth. This isoform may function to critically maintain cortical renal function in the immediate newborn period, when renal hemodynamics are changing rapidly. Because the renal expression of eNOS differs from nNOS, further investigation of these isoforms is needed to identify the mechanisms producing their developmental regulation and their roles during postnatal renal development.

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