Ontogeny of neuronal nitric oxide synthase, NOS I, in the developing porcine kidney

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Solhaug, Michael J., Xui Q. Dong, Raymond D. Adelman, and Ke-Wen Dong. Ontogeny of neuronal nitric oxide synthase, NOS I, in the developing porcine kidney. Am J Physiol Regulatory Integrative Comp Physiol 278: R1453–R1459, 2000.—To determine if the developing kidney differs from the adult in the expression of the neuronal nitric oxide synthase, NOS I, these experiments measured mRNA gene expression by RNase protection assay and protein content by Western blot of NOS I in piglets at ages newborn and 3, 7, 10, 14, and 21 days and adult pigs. Whole kidney NOS I mRNA was greatest at birth and decreased progressively during renal maturation to adult levels. NOS I protein content paralleled this developmental pattern. Cortical NOS I protein was equivalent in newborn and 14-day-old piglets and was greater at both ages than the adult. Medullary NOS I protein was relatively greater than cortical in both immature ages and decreased from a peak at birth to adult levels. We conclude the following. 1) During postnatal maturation, renal NOS I mRNA and protein content show a pattern that is developmentally regulated. 2) This developmental pattern of NOS I after birth may, in part, contribute to the enhanced functional role of NO during renal maturation.

NITRIC OXIDE (NO) has an enhanced functional role in the postnatal developing kidney compared with the adult. In adult renal function, it is well established that NO is an important regulator of renal hemodynamics (12), in particular, modulating the vasoconstrictor actions of ANG II (28, 31). Recent studies in the immature kidney demonstrate that under physiological and pathophysiological conditions, NO critically supports not only renal blood flow but also glomerular filtration rate (GFR), counterbalancing highly activated vasoconstrictor mechanisms, such as the renin-angiotensin system (2, 32, 34, 36). The mechanisms producing this enhanced participation of NO during postnatal renal maturation may involve differences from the adult in the renal localization and content of the NO synthesizing enzyme nitric oxide synthase (NOS).

Three isoforms of NOS have been localized in the adult kidney, including neuronal NOS or NOS I (1). Immunocytochemical and in situ hybridization techniques have identified the macula densa as a principal site of NOS I in the adult kidney (1, 17, 37). Additionally, in the adult kidney, other nephron segments also express NOS I, including medullary thick ascending limb (23) and inner medullary collecting duct (IMCD; 20, 21, 26, 27, 32, 40). Medullary NOS I may have greater expression and enzymatic activity than that found in the cortex (26, 41). NO, produced by the NOS I isomor in the macula densa, has been shown to participate as an intrarenal vasoregulatory factor in adult rats (14, 15). Although in the adult kidney NOS I has been extensively studied and the renal hemodynamic functional importance of NO during renal development is known, the quantification of NOS I in the developing kidney has not been completely analyzed.

NOS intrarenal localization has been performed in the developing rat (10) and piglet (35) kidney. NOS I is identified in the earliest stages of nephrogenesis in the area fated to become the macula densa and marks the development of this tubular segment throughout the formation of the nephron (10, 35). The developing kidney also demonstrates an abundant tubular localization that differs from the adult (35). Fischer et al. (10) attempted to quantify NOS I in the rat kidney at ages 2, 6, and 15 days of age, but not in the adult, by evaluating the fraction of NOS-positive glomeruli and the number of NOS-positive macula densa cells. This technique demonstrated maximal NOS I expression on day 6 decreasing with age. However, more precise methods are available to quantifiably demonstrate the renal developmental pattern expression of the mRNA and protein of this enzyme. Also, an analysis of the complete maturational spectrum of NOS I must compare the immature kidney to the adult. Taken together, these studies show that NOS I demonstrates a differing developmental localization pattern in the immature kidney compared with the adult (10, 35), and further suggest that there may be quantifiable differences of NOS I between the developing and adult kidney (10).

This information was the basis for our hypothesis that the critical participation of NO in the developing kidney is due, in part, to differences from the adult in the content and regulation of NOS I during postnatal development. The purpose of our studies was to measure renal NOS I mRNA gene expression and protein content throughout the postnatal maturational spectrum. The experiments were performed in developing piglets ages newborn (2 h) and 3, 7, 10, 14, and 21 days and adult pigs. Whole kidney NOS I mRNA gene expression was measured in all groups by RNase protec-
tion assay using a species-specific porcine NOS I cDNA. Whole kidney NOS I protein content was measured by Western blot analysis, using an anti-porcine NOS I antibody, in all age groups. Additionally, NOS I protein content was measured in cortex and medulla by Western blot in newborn and 14-day-old piglets and adults.

**MATERIALS AND METHODS**

**Animals.** All experiments were performed on mixed breed swine obtained from local vendors. Kidneys of preweanling piglets 2 h and 3, 7, 10, and 14 days of age were harvested on arrival and from weaned piglets at 21 days and adults 70 days of age after 24 h.

**Tissue preparation.** After pigs are killed with Euthasol (Delmarva Laboratories, Midlothian, VA), 50 mg/kg iv, kidneys are immediately removed from the animals (piglets and adult pigs) and coronally sectioned, conserving the corticomedullary architecture from the lower and upper pole and midkidney regions, and processed for whole kidney analysis. Separation of cortex and medulla is accomplished using a dissecting microscope. The prepared tissue is then immediately frozen in liquid nitrogen and stored at −80°C.

**Creation of effective molecular probes for NOS.** A porcine species-specific and NOS isoform-specific probe was developed using RT-PCR and subcloning techniques. The subcloning of species and isoform-specific cDNA was a critical step in most effectively analyzing intrarenal NOS mRNA gene expression in the developing piglet and adult pig. To subclone the cDNA of the porcine NOS I isoform, published sequences of the full-length cDNAs were compared between human and rodent species. Computer analysis (GCG computer program, Wisconsin University) of published cDNA sequences of human, rat, and mouse NOS I gene was performed, and a pair of primers was designed on the basis of portions of the sequences with the highest homology within the three species and the greatest difference among the other individual NOS isoform genes. The sense primer (5′-TCAAGGTCGACTTCGAG-3′) is located at +1586, and the anti-sense primer (5′-CGTCCAGTGACCTGACGATG-3′) is located at +1940 according to human NOS I gene sequence. An adult porcine kidney sample, processed as described in RNase protection assay, was used for the source of RNA for RT-PCR. The RT-PCR product for NOS I was then purified by Geneclean (Bio 101) and inserted into pGEM-T vector (Promega, WI).

**RNase protection assay.** The RNase protection assay was performed in piglets ages newborn, 3, 7, 10, and 14 days of age and adults. The porcine NOS I isoform-specific cDNA of interest, generated and subcloned as described above, is linearized with Nco I and Spe I, and riboprobes are synthesized using T7 and T3 RNA polymerase and labeled with [32P]UTP (NEN, Boston, MA) to a high specific activity [1 × 109 counts/min (cpm)/µg]. RNA probe (antisense) and sense RNA are generated. A rat cyclophilin gene (a housekeeping gene) is used as an internal control to normalize the results from each sample. The sense RNA for each construct is synthesized at low specific activity (106 cpm/µg) and used to generate a standard curve for quantification of the RNA sample. About 700 pg of probe is mixed with 30–150 µg total RNA to a final volume of 25 µl hybridization solution (4.0 M guanidium thiocyanate, 0.1 M EDTA, 30 mM NaCl, 3.0 mM Na-citrate; pH 7.0). For the standardized curve, the same amount of probe is mixed with sense RNA. The mixture is hybridized overnight (~12 h) at 30°C, then treated with proteinase K for 15 min at 45°C. After phenol-chloroform extraction, the RNA and cyclophilin samples are precipitated with ethanol, dissolved in gel loading buffer, and run through an agarose gel. The gel is dried and exposed overnight to X-ray film at −70°C with an enhancing screen. The radioactivity of the protected bands of NOS I (360 bases) and cyclophylin mRNA are quantitated by Molecular Dynamic Phosphorimager (Image Quant software program). The amount of radioactivity in each sample determined by the PhosphorImager was compared with the amount of reference RNA calculated by regression analysis.

**Western blot analysis.** Western blots are performed on whole kidney homogenates or from cortex and medulla tissue as described above. Whole kidney Western blot was performed on piglets ages newborn and 3, 7, 10, 14, and 21 days of age and adult pigs. Separate experiments performed Western blot on the cortex and medulla in piglets newborn and 14 days of age and adult pigs. These age groups were selected for this study to provide a representative analysis of the postnatal developmental spectrum. The 14-day-old piglet was also chosen because the animals are weaned at this age, making them available for other studies. The homogenates are separated on 7.5% denaturing sodium dodecyl sulfate-polyacrylamide gels. The proteins are then blotted onto nitrocellulose (Hy-Bond, Amersham, Arlington Heights, IL) by electroblot for 2 h. The blots are blocked overnight at 4°C with 6% nonfat dry milk in Tris-saline buffer. The blots are then washed twice in Tris-saline buffer and then incubated with an NOS I porcine isoform-specific antibody (anti-rabbit, Alexis Biochemicals, San Diego, CA) at a dilution of 1:4,000 in Tris-saline buffer for 2 h at room temperature. The blots are washed four times in Tris-saline buffer and incubated with horseradish-peroxidase-conjugated rabbit immunoglobulin (Amersham) antibody for 1 h at room temperature. Finally, the blots were washed four additional times in Tris-saline buffer, and the specific proteins are detected by enhanced chemiluminescence (Amersham). Negative controls are performed as above without the primary antibody and on porcine liver, which does not contain NOS I.

**Statistical analysis.** All assays were repeated three times for each animal. Results were analyzed by analysis of variance and expressed as means ± SE with statistical significance at P < 0.05.

**RESULTS**

Preparation of a vector for mRNA probe from porcine NOS I cDNA for RNase protection assay. With the use of the NOS I isoform-specific primers, the predicted 360-bp cDNA fragment was obtained. When NOS II and NOS III specific primers were used, two expected PCR products were obtained: 230 bp for NOS II and 260 bp for NOS III, as shown in Fig. 1A. Subcloning and subsequent analysis showed the porcine mRNA fragment to have high homology with the respective human NOS I cDNA, 76% homology (Fig. 1B), confirming the successful manufacture of this probe for studies.

RNase protection assay determination of whole kidney NOS I gene expression. The postnatal pattern of steady-state quantification by RNase protection assay of whole kidney NOS I mRNA is shown in Fig. 2. All stages of postnatal renal maturation up to 21 days had significantly greater NOS I mRNA than the adult. The newborn kidney demonstrated the greatest NOS I mRNA gene expression with 158 ± 6 pg NOS I/30 µg total RNA. This compares to the adult level of 24 ± 8 pg NOS I/30 µg total RNA. With increasing age, NOS I mRNA gene expression in the immature ages progres-
sively declined to $51 \pm 9$ pg NOS I/30 µg total RNA in the 21-day-old piglet.

Assessment of whole kidney NOS I protein content by Western blot. Whole kidney NOS I protein content by Western blot is shown in Fig. 3. Renal NOS I protein shows a maturational pattern similar, but not identical, to that demonstrated by NOS I mRNA. NOS I protein content decreases more abruptly than mRNA expression, dropping rapidly from newborn to 3 days of age. Thereafter, the protein content progressively decreases in parallel with mRNA expression to adult levels. All immature ages, except the 21-day-old piglet, contain significantly greater renal NOS I protein than the adult. Whole kidney NOS I protein is greatest in the newborn, $15.1 \pm 0.6$ RAU. NOS I protein progressively decreases with age to the lowest level in the adult, $2.4 \pm 0.8$ RAU.

Assessment of cortical and medullary NOS I protein content by Western blot. In Fig. 4, cortical and medullary NOS I also demonstrate distinct developmental patterns during postnatal renal maturation. Medullary NOS I protein content was greater than cortical at all ages studied: newborn, 14-day-old piglets, and adults. Medullary NOS I, greatest in the newborn at $8.8 \pm 0.3$ RAU, decreases with age in a pattern that parallels whole kidney NOS I to the lowest level at the adult, $1.3 \pm 0.4$ RAU. Cortical NOS I remains at the same level from newborn to 14 days of age, $1.5 \pm 0.2$ and $1.7 \pm 0.4$ RAU, respectively, then declines to the adult level, $0.5 \pm 0.3$ RAU.

DISCUSSION

These experiments are the first specific characterization of renal NOS I, both whole kidney and corticomedullary, throughout the entire postnatal developmental spectrum. Whole kidney NOS I mRNA quantified by RNase protection assay and protein content determined by Western blot follow a parallel pattern from the newborn piglet to the adult pig. The greatest whole kidney NOS I levels are seen in the newborn, then progressively decrease with age to the lowest values, which are in the adult. Localization studies in developing rats (10) and piglets (35) suggest that NOS I is more abundant in the immature kidney than in the adult. In the only previous characterization of NOS I in the developing kidney, Fischer et al. (10) also attempted to quantify postnatal renal NOS I in the rat at ages 2, 6, and 15 days of age by evaluating the fraction of NOS-positive glomeruli and the number of NOS-positive afferent arterioles.
I-positive macula densa cells identified by in situ hybridization. By this technique, NOS I was greatest in the 6-day-old rat kidney. However, whole kidney NOS I was not evaluated. This quantification method counted the number of NOS-positive glomeruli identified by the NADPH diaphorase technique, which labels all three NOS isoforms and the number of macula densa cells per glomeruli containing in situ hybridization-identified NOS I mRNA. Although this evaluation suggested that NOS I differs during postnatal renal development, it only examined a sampling of glomeruli-associated NOS I cells and, therefore, was not an accurate determination of whole kidney NOS I. Furthermore, the developmental pattern described in these studies was incomplete, because immature animals were not compared with the adult. In our studies using specific quantification techniques and comparing the measurements for the entire period of postnatal changes, whole kidney NOS I mRNA and protein show a progressive decline in abundance with age throughout maturation. NOS I protein content decreases more abruptly from newborn to 3 days of age, but thereafter progressively decreases in parallel with mRNA expression. Whole kidney NOS I protein content in the 21-day-old piglet did not differ from the adult. This similar NOS I protein content could be due to the disappearance of the zone of nephrogenesis in the cortex of the more mature 21-day-old kidney, the continued decline in medullary NOS I from the 14-day-old level shown in these studies, and the regression of NOS I tubular content as the developing nephrons mature (36). The overall pattern of whole kidney NOS I after birth demonstrates that renal NOS I is developmentally regulated from birth to the adult.

The corticomedullary pattern of NOS I also appears to be developmentally regulated after birth. Our studies demonstrate a differential developmental pattern between cortex and medulla. Cortical levels of NOS I protein in the younger age groups, newborn and 14-day-old piglets, although equivalent at these immature ages were both greater than in the adult. The reason that the cortical levels in the two immature age groups remain the same is not known. The comparable cortical levels between newborn and 14 days of age in the piglet, both of which are greater than the adult, could be accounted for by the balance of continued production of NOS I by nephrogenesis in the outer cortex and the changes in cortical NOS I content in the maturing nephron. As in most mammals, with the human being an important exception, in swine, nephrogenesis continues after birth and up to 14–18 days of age. NOS I is found in the earliest stages of the newly forming NOS I-positive macula densa cells identified by in situ hybridization. By this technique, NOS I was greatest in the 6-day-old rat kidney. However, whole kidney NOS I was not evaluated. This quantification method counted the number of NOS-positive glomeruli identified by the NADPH diaphorase technique, which labels all three NOS isoforms and the number of macula densa cells per glomeruli containing in situ hybridization-identified NOS I mRNA. Although this evaluation suggested that NOS I differs during postnatal renal development, it only examined a sampling of glomeruli-associated NOS I cells and, therefore, was not an accurate determination of whole kidney NOS I. Furthermore, the developmental pattern described in these studies was incomplete, because immature animals were not compared with the adult. In our studies using specific quantification techniques and comparing the measurements for the entire period of postnatal changes, whole kidney NOS I mRNA and protein show a progressive decline in abundance with age throughout maturation. NOS I protein content decreases more abruptly from newborn to 3 days of age, but thereafter progressively decreases in parallel with mRNA expression. Whole kidney NOS I protein content in the 21-day-old piglet did not differ from the adult. This similar NOS I protein content could be due to the disappearance of the zone of nephrogenesis in the cortex of the more mature 21-day-old kidney, the continued decline in medullary NOS I from the 14-day-old level shown in these studies, and the regression of NOS I tubular content as the developing nephrons mature (36). The overall pattern of whole kidney NOS I after birth demonstrates that renal NOS I is developmentally regulated from birth to the adult.

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The developing kidney contained a greater proportion of medullary NOS I relative to the cortex than the adult. Although renal medullary function of NO has been examined in the adult (21, 22, 41), the functional significance of greater amounts of NOS I in medulla in the immature kidney is not known. Further investigations are needed to clarify the role of NO in medullary tubular function during postnatal renal development. Also, because only two age groups were studied, newborn and 14-day-old piglets, further changes may take place in corticomedullary NOS I during the period of renal maturation.

Whole kidney and differential corticomedullary NOS I follows a developmental pattern in which regulation of NOS I expression changes with age. Although NOS I has been identified in the early morphogenesis of the nephron, particularly in the macula densa and other tubules (10, 35), our studies are the first to identify the complete postnatal developmental pattern of NOS I in the kidney. The changes of renal NOS I expression with increasing postnatal age strongly suggest that regulatory mechanisms are producing this sequential pattern. Interestingly, immediately after birth, NOS I protein decreases more rapidly than mRNA expression. The reasons for the differing pattern between NOS I protein and mRNA expression immediately after birth are not known. It is possible that in the early postnatal period, NOS I protein and mRNA are influenced by different regulatory mechanisms. The developmental regulation of NOS I during postnatal renal maturation has not been studied. However, this isoform has been characterized in the development of other organs. NOS I undergoes developmental expression in the central nervous system (8, 19, 20, 25) and lung (24). The mechanisms specifically regulating renal NOS I expression after birth have not been studied. The unique complex structural organization of the NOS I gene provides potential regulatory mechanisms during development. The expression regulation of NOS I is possible through different transcriptional units containing alternate promoter sequences potentially resulting in multiple NOS I mRNA transcripts (3, 5, 7, 11, 18). Additionally, NOS I may undergo posttranscriptional regulation (11). Hormonal and vasoactive factors may play a role in the postnatal regulation of renal NOS I. Rat brain NOS I transcription is regulated by glucocorticoids (39) and estradiol (6), both of which are in abundance in the neonate and change with postnatal age. PGs, specifically high levels of PGE₂, upregulate NOS I mRNA, protein, and activity in the neonatal rat brain (9). The renin-angiotensin system presents as a particularly attractive potential NOS I regulating factor during development. All components of the renin-angiotensin system exhibit a postnatal renal pattern that is similar to that revealed in these studies of NOS.
I, with the greatest amounts in the newborn, then decreasing with age (10, 38). Colocalization studies suggest an interactive relationship between NOS I-containing cells and renin-producing cells in the developing rat kidney (10). NO and ANG II demonstrate functional interactions that control renal hemodynamics in adult kidneys (28, 31) and during postnatal development (32, 36). The functional counterregulation of NO against ANG II during development maintains the maturation of GFR and renal blood flow (32, 36), as well as regulates renin release (36). In adult animal models, NOS I and renin are regulated interdependently, demonstrating concordant changes with various stimuli, including dietary salt and renal perfusion (4, 29). Recent studies support the regulation of NOS I by ANG II through AT1 receptors. The AT1 receptor antagonist Losartan downregulates NOS I protein expression in both cortex and medulla in the normal rat kidney (26). Finally, the ANG II AT receptor subtypes AT1 and AT2 undergo postnatal changes in expression that complement the pattern of NOS I protein and mRNA expression. The AT2 receptor, which predominates in the fetal kidney, rapidly decreases and is virtually absent after 14 days of age in neonatal rats. In contrast, AT1 receptors progressively increase after birth (38). This differential receptor pattern may participate in the early differences between NOS I protein content and mRNA expression. The similarity of the postnatal developmental pattern between NOS I and the renin-angiotensin system, an interdependent colocalization of NOS-1- and renin-containing cells, the functional interplay of NO with ANG II during renal maturation, and the coregulatory interaction between NOS I and elements of the renin-angiotensin system in adults all suggest an important developmental regulatory interaction for these factors. Further investigation is needed to identify the factors that regulate NOS I during postnatal renal development and their mechanisms of action.

In our experiments, NOS I expression in whole kidney, both for mRNA and protein, was greater at all immature ages compared with the adult. This quantitative expression pattern is consistent with studies showing that NO participates to a greater degree in postnatal renal function than the adult. Relative to the adult, the newborn has a lower renal blood flow (RBF), maintained by a high renal vascular resistance (RVR), resulting in a lower GFR. After birth, these functional elements change rapidly in the postnatal period. Most of the increases in RBF and GFR are due to the progressive decline in RVR, such that with increasing age, renal hemodynamics synchronously change to achieve adult capability. It is during the early postnatal period that the vasodilator function of NO is most critical. To date, NO is the only such vasodilator identified during this time of renal hemodynamic change. Studies demonstrate that NO is an important vasodilator during this period in the developing kidney, counterbalancing highly activated vasoconstrictors, such as the renin-angiotensin system through ANG II (32, 36). Furthermore, NO supports RBF and GFR during hypoxia (2, 36). The increased NOS I could account for the enhanced NO activity in the immature kidney. NO, produced by the NOS I isoform in the macula densa, has been shown to participate as a vasoregulatory factor in adult rats. NOS I in the macula densa, by virtue of extensive contacts with portions of the afferent arteriole and, to a lesser degree, the efferent arteriole, functions as a communicating regulator of glomerular arteriolar resistance (14, 15). Thus the NOS I developmental pattern shown in these studies provides a possible mechanism for the enhanced vasodilator role of NO during postnatal renal maturation.

In summary, NOS I expression demonstrates a distinct developmental pattern in the postnatal kidney. Whole kidney NOS I mRNA gene expression and protein content are greatest in the newborn, decreasing with age though the period of renal maturation to the lowest levels in the adult. Cortical and medullary NOS I protein is differentially expressed after birth. Compared with the adult: 1) the developing kidney contains greater amounts of NOS I in both cortex and medulla and 2) medullary NOS I, which is greatest at birth and progressively decreases with age, is relatively greater than cortical protein expression during development.

In conclusion, renal NOS I undergoes postnatal regulation, both whole kidney and differential cortical-medullary, and this developmental pattern may provide a mechanism to explain the participation of NO during the period of renal maturation.

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