

# Age dependent expression and distribution of nitric oxide (NO) synthase isoforms in the ovine kidney

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## ABSTRACT

The aim of the present study was to measure intrarenal spatial and temporal localization of all three nitric oxide synthase (NOS) isoforms in the developing ovine kidney. Reverse transcriptase-polymerase chain reaction (RT-PCR), Western Blot analyses, and *in situ* hybridization techniques were performed for NOS I - III isoforms in renal tissue obtained from sheep aged ~24 h, one, three, six, and 12 weeks postnatally (N = 3). RT-PCR performed on cortical and medullary kidney tissue revealed the presence of all three NOS isoforms from day one to 12 weeks postnatally. NOS I and NOS II mRNA levels were greater in cortex compared to medulla during the first three weeks whereas NOS III mRNA levels were predominantly transcribed within the medulla. In all NOS isoforms, there was a decrease in cortical mRNA levels after three to six weeks. Protein levels confirmed the presence of all three NOS isoforms over the first three months of postnatal life. By demonstrating NOS isoform transcripts to be more abundant in the early postnatal period, these findings may provide insight into the age dependent role of NO in modulating kidney function during ontogeny.

**Keywords:** Postnatal; Kidney; Newborn; iNOS; eNOS; nNOS; Nitric Oxide

## 1. INTRODUCTION

The endothelial derived relaxing factor, nitric oxide (NO), is a gaseous free radical that functions as an endogenous mediator of a variety of physiological processes. Through its effects on vascular smooth muscle, NO is involved in the maintenance of organ blood flow and blood pressure regulation. In addition, NO modulates renal hemodyna-

mics and function, possesses immunomodulatory as well as cytotoxic effects, and may act as a neurotransmitter in the central nervous system. NO production is regulated by NO synthases (NOS), of which there are three distinct isoforms: neuronal NOS (nNOS, or NOS I), inducible NOS (iNOS, or NOS II), and endothelial NOS (eNOS, or NOS III). Numerous transcriptional, translational, and post-translational controls govern the timing, magnitude, and spatial distribution of NO release [1].

In previous studies carried out in conscious, chronically instrumented lambs, we provided evidence that some of the physiological effects of NO are developmentally regulated [2-4]: Administration of the L-arginine analogue, N<sup>G</sup>-nitro-L-arginine methyl ester, L-NAME, which prevents NO production from NOS isoforms, is associated with age-dependent effects on renal hemodynamics and function: After treatment with L-NAME, there is a marked increase in renal vascular resistance (RVR), which is greatest at one-week, and least at six weeks, postnatally [3]. Also, after administration of L-NAME to one-week old lambs, glomerular filtration rate (GFR) and filtration fraction (FF) decrease by ~50% whereas in six-week old lambs, GFR and FF remains constant after L-NAME [5]. In lambs aged ~six weeks, proximal fractional Na<sup>+</sup> reabsorption decreases after L-NAME administration, resulting in a prompt natriuresis; in contrast, there are no effects on proximal fractional Na<sup>+</sup> reabsorption at one-week. These findings demonstrate that, in conscious lambs, endogenously produced NO modulates glomerular and tubular function in an age-dependent manner [5].

The renal distribution of NOS I and III also appears to be developmentally regulated, at least in some species: For example, renal NOS I [6] and NOS III mRNA [7] in piglets and adult pigs showed developmental patterns of expression. Fischer *et al.* [8] measured the postnatal

development of NOS I expression in the rat kidney and found that it follows a cortico-medullary pattern ranging from single cell expression in the immature nephron to its full presence in the *macula densa* of nephrons in the mature kidney [8]. Similarly for NOS III, Hanm *et al.* [9] demonstrated a unique corticomedullary pattern in the newborn rat kidney which was distinct from that observed in the adult rat kidney.

To date, any age-dependent changes in NOS expression in the ovine kidney which could explain the aforementioned physiological findings have not been investigated. Moreover, there have been no previous investigations into the developmental patterns of renal expression for all three NOS isoforms. Therefore, the aim of the present experiments, was to measure mRNA and protein expression for NOS I, II and III from day one to 12 weeks of post natal life in the developing ovine kidney. In addition, NOS transcripts were localized using *in situ* hybridization techniques in order to evaluate the localization of the three NOS isoforms during this post natal period.

## 2. METHODS

Sheep (*ovis aries*) were obtained from a local vendor (Woolfitts, Acres, Olds, Alberta, Canada) and euthanized in accordance with the "Guide to the Care and Use of Experimental Animals" provided by the Canadian Council on Animal Care and with the approval of the Animal Care Committee of the University of Calgary. There were three experimental protocols which evaluated: 1) mRNA expression using RT-PCR, 2) immunoreactive protein levels using Western Blot analyses, and 3) mRNA localization using *in situ* hybridization techniques. Each protocol was carried out in different tissue and from different groups of animals as detailed below.

### 2.1. Experimental Protocol I

#### 2.1.1. Tissue Preparation

Sheep of either sex and aged 24 h, one, three, six, and twelve weeks of post natal life (N = 3 at each age group) were humanely euthanized (see **Table 1** for demographic details). Either kidney was rapidly excised and divided

**Table 1.** Demographic data.

Age (weeks)	n	Body weight (kg)	Kidney weight (g)
24 h	3	5.2 - 5.7	14.6 - 17.4
One	3	5.5 - 6.8	15.6 - 21.0
Three	3	6.7 - 11.6	18.4 - 32.0
Six	3	12.4 - 17.2	33.6 - 43.7
Twelve	3	23.6 - 44.5	41.0 - 73.5

sagittally along pyramidal borders. Cortical and medullary regions were separated, rinsed in sterile, phosphate-buffered saline (PBS) to remove blood, blotted on filter paper, weighed, and snap frozen in liquid nitrogen for storage at  $-80^{\circ}\text{C}$  until RNA extraction, as detailed below.

#### 2.1.2. RNA Extraction and RT-PCR

The TRIspin method was used to measure total RNA [10]: Frozen tissue was powdered in a liquid nitrogen-cooled Braun Mikro-Dismembrator Vessel, TRIZOL reagent ( $1\text{ mL}\cdot 100\text{ mg}\cdot\text{tissue}^{-1}$ ) was added to the homogenate, and the sample incubated at room temperature to allow for complete dissociation of nucleoprotein complexes and then combined with chloroform (0.3 mL). Samples were vortexed and centrifuged at 12,000 rpm for 15 min at  $4^{\circ}\text{C}$ , and the upper layer transferred and ethanol added. The precipitate was obtained, solubilized, and total RNA isolated using the RNeasy<sup>®</sup> Total RNA Kit (Qiagen, Chatsworth, CA). The yield of RNA was quantified fluorometrically using a reagent (Sybrgreen II; Mandel, Guelph, Ontario, Canada) [11], over the linear range  $100$  to  $800\text{ ng}\cdot\text{mL}^{-1}$  and stored at  $-80^{\circ}\text{C}$  until analyzed.

Total RNA ( $1\text{ }\mu\text{g}$ ) was reverse-transcribed (RT) into cDNA using the Reverse Transcription Kit with random primers (Omniscript RT Kit; Qiagen, Mississauga, Ontario, Canada), according to the manufacturer's protocol. The newly synthesized cDNA, stored at  $-20^{\circ}\text{C}$ , was amplified by polymerase chain reaction (PCR) [12-14] using sheep-specific primers designed using Primer Express Software (Applied Biosystems). For details of gene of interest, primer sequences, base pairs, sources, annealing temperature and cycle numbers, see **Table 2**. This PCR protocol was designed to result in product yields within the linear range of the PCR amplification. All samples were reverse transcribed and subjected to PCR simultaneously to avoid any potential variation in the efficiency of the procedures. PCR product was purified using a Qiagen PCR purification kit and subsequently verified by sequencing. PCR products were visualized by electrophoresis of  $20\text{ }\mu\text{L}$  of reaction mixture on a 2% agarose gel at  $60\text{ V}/\text{cm}$  in TAE buffer. Gels were stained with ethidium bromide, destained in distilled water, photographed and analyzed using a BioRad Gel-Doc (BioRad Corporation) with Discovery Series Quantity One 1-D Analysis software (version 4.5.1). Integrated density values were normalized to the house-keeping gene  $\beta$ -actin, to yield a semi-quantitative assessment of individual transcript levels. In another system, recent direct comparisons of mRNA levels using the rigorously controlled semi-quantitative methods described above to real-time PCR results (BioRad iCycler) have revealed nearly identical outcomes (Hart DA and Reno CR; unpublished observations).

**Table 2.** Primer sequences and PCR conditions.

Primer	Sequence	Base pairs	Annealing Temperature (°C)	Cycle	Source
$\beta$ -actin	F: GCT ACA GCT TCA CCA CCA CG	304	60	22	GenBank OAU39357
	R: ACC GTG TTG GCG TAG AGG TC				
NOS I	F: AGC TCG AGA CCC TCA GTC AG	296	65	32	GenBank OAU76738
	R: GTC TCC AGT CTT GAG TTG GC				
NOS II	F: CAA GCA CCA CAT TGA GAT CC	397	65	34	GenBank AF223942
	R: TGG CAG TCG CTT ATC CTT GA				
NOS III	F: ATG TCC ACA AGA AGC GGG TCT CAG	296	65	36	GenBank OAU76739
	R: GCG ATG CTC ATC GGT CCA GTT ACT				

## 2.2. Experimental Protocol II

Protein was isolated and quantified using a RCDC BioRad Protein Reagent Kit (BioRad Laboratories Ltd, Mississauga, ON) on a cuvette compatible Cary 300 UV-Visible Spectrophotometer (Agilent Technologies, Mississauga, ON). Electrophoresis and a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to separate proteins with gels made from 30% Protogel (National diagnostics, Diamed, Mississauga, ON) onto 0.45  $\mu$ m nitrocellulose membranes (BioRad Laboratories Ltd, Mississauga, ON). There is a high sequence homology (90% - 95%) for the same NOS isoform across species [15,16]. In the present study, proteins were exposed to rabbit polyclonal antibodies (Santa Cruz, USA) against human NOS I (epitope corresponding to amino acids 2 - 300 mapping at the N-terminus, 155 kDa), NOS II (amino acid 1 - 20 mapping at the N-terminus, 130 kDa) and NOS III (amino acids 2 - 160 mapping at the N-terminus, 140 kDa) at a dilution of 1:400 in 5% skimmed milk PBST and subsequently, goat anti-rabbit IgG HRP-conjugated secondary antibody (1:1000). Immunoreactive proteins were visualized using chemiluminescence reagents (Immobolin Western Chemiluminescent HRP Substrate) and blots analysed using densitometric analysis software (Discovery Series Quantity One, version 4.5.1). GAPDH was used as loading control to normalize NOS expression. To verify that GAPDH was not regulated by development, the level of each NOS isoform was also normalized for the amount of protein loaded.

## 2.3. Experimental Protocol III

### 2.3.1. Tissue Preparation

Upon euthanization as described for Protocol I, the kidney was rapidly excised and the renal capsule was removed. Kidneys were then cut in coronal sections and

dissected into columns which were immediately embedded in Fisher Histo Prep frozen tissue embedding media (Fisherbrand Scientific), and frozen at  $-80^{\circ}\text{C}$ . Kidney tissue samples were obtained at the same ages as for Protocol I.

### 2.3.2. Riboprobe Synthesis

Subcloning of unique PCR fragments for NOS I, II and III, was performed using PCR 2.1-TOPO (Invitrogen, Carlsbad, CA). Sequences of forward and reverse primers for the three NOS isoforms are shown in **Table 2**. The resulting plasmids were sequenced using the DNA Services Core Facility of the University of Calgary, to confirm orientation and heterogeneity of nucleotide sequences and to rule out cross talk between riboprobes, using NOS II sense as a negative control. Bacteria were cultured and selected after growth on agar plates containing ampicillin, at  $37^{\circ}\text{C}$  overnight. Bacterial colonies were further amplified and plasmids extracted using QIAprep Spin Miniprep Kit (Qiagen). Probe synthesis was then performed following linearization of the plasmid with BamH1, and *in vitro* transcription for synthesis and digoxigenin (DIG)-labelling using T7 RNA polymerase. Evaluation of BLAST searches for each isoform sequence confirmed the specificity of the probes.

### 2.3.3. In Situ Hybridization

Cryosections of 10  $\mu$ m were cut onto superfrost plus slides (Fischer), and heated to  $65^{\circ}\text{C}$  for 2 h, rinsed in PBS, and fixed in 4% paraformaldehyde. Sections were then permeabilized in proteinase K ( $10 \mu\text{g}\cdot\text{mL}^{-1}$  in PBS) at  $37^{\circ}\text{C}$  for 30 min, post-acetylated in 200 mL of 0.1 M TEA with 1 mL of acetic anhydride, and twice rinsed in standard saline citrate (SSC). The transcribed riboprobe was verified using Dot-blot analysis and concentrations were determined using spectrophotometry (Ultrospec™ 2100 *pro* UV/Visible Spectrophotometer). Approxima-

tely  $300 \text{ ng}\cdot\text{mL}^{-1}$  of probe was placed in hybridization buffer (formamide 100%,  $20 \times \text{SSC}$ ,  $50 \times \text{Denhardt's}$  solution (Sigma)), denatured salmon sperm DNA ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ), yeast tRNA ( $20 \text{ mg}\cdot\text{mL}^{-1}$ , Roche), 50% dextran sulphate) and immersed onto tissue sections at  $48^\circ\text{C}$  for 16 h. Negative controls consisted of tissue sections treated with RNase A pre-hybridization buffer only, hybridization buffer without probe, and hybridization buffer containing NOS III sense probe. After rinsing in RNase A and blocking via sheep serum, antibody solution ( $1/500$  anti-DIG antiphosphatase, Roche) was applied and sections incubated at  $28^\circ\text{C}$  for 3 h. After rinsing in PBS, sections were processed for detection by the addition of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Following the chromogenic reaction, slides were rinsed in  $\text{ddH}_2\text{O}$ , followed by eosin Y counterstaining, stepwise dehydration in graded ethanol, clearing in Citri Solv (Fisherbrand Scientific), and mounted using Cytoseal XYL (Fisherbrand Scientific). Sections were viewed using a microscope (Zeiss Axiostar plus; Zeiss Instruments.) with the assistance of a renal pathologist.

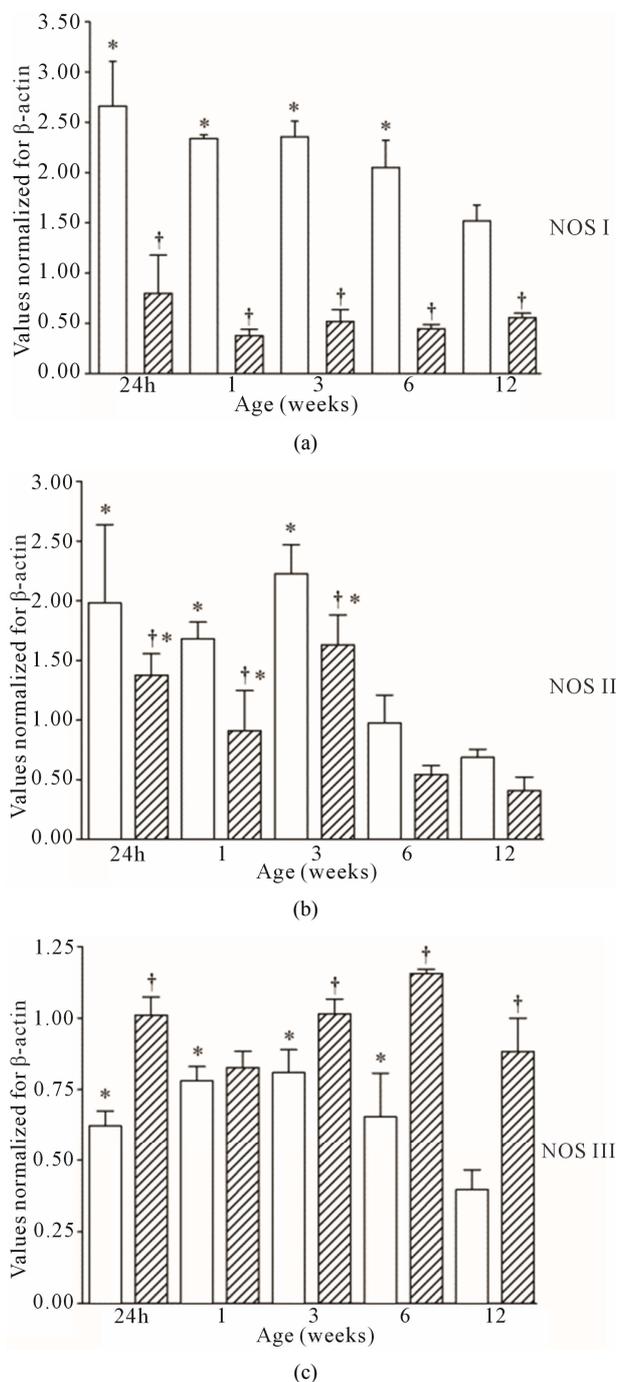
## 2.4. Data Analyses

For protocol I and II, measurements were averaged and statistical analyses carried out using one-way ANOVA followed by Holm-Sidak multiple comparisons, using a confidence interval of 95%. Data are presented as mean  $\pm$  SD. For protocol III, photographs ( $n = 248$ ) were taken from tissue sections ( $n = 192$ ) and evaluated in a blinded qualitative analysis by two independent scorers, according to previously published methods [17]. Values represent averages of scoring as absent (-), low (+), moderate (++) , and high (+++).

## 3. RESULTS

### 3.1. Experimental Protocol I

All three NOS isoforms were expressed in the cortex and medulla of the developing ovine kidney over the first three months of post natal life. NOS I mRNA was higher in the cortex as compared to the medulla at all age groups (**Figure 1(a)**). Cortical mRNA levels decreased from six to 12 weeks of age whilst medullary levels remained low. NOS II mRNA levels were higher in cortex than medulla during the first three weeks post natally; both cortical and medullary levels decreased after three weeks (**Figure 1(b)**). Medullary levels fluctuated in a defined pattern. NOS III mRNA was predominantly transcribed within the medulla, with higher levels as compared to the cortex at 24 h, and again from three to 12 weeks (**Figure 1(c)**). Cortical mRNA levels decreased after six weeks. Representative images ( $n = 3$ ) of NOS

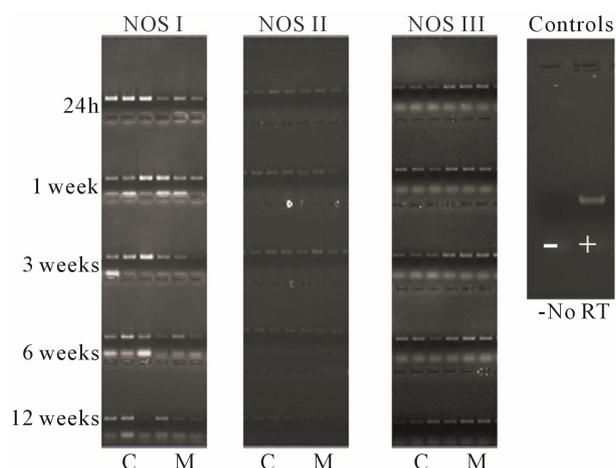


**Figure 1.** NOS mRNA expression cortex, open bars; medulla hatched bars. † $p < 0.05$  compared to cortex; \* $p < 0.05$  compared to 24 h.

I-III in cortex and medulla from 24 h to 12 weeks are presented in **Figure 2**. Results for non-reverse transcriptase controls were negative indicating undetectable levels of genomic DNA.

### 3.2. Experimental Protocol II

Immunoreactive proteins for all three NOS isoforms



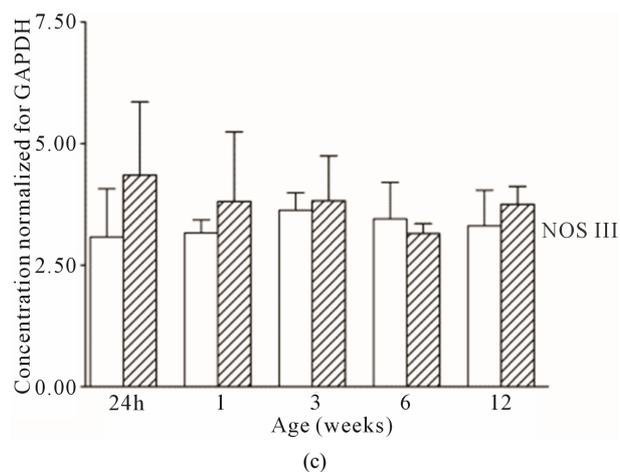
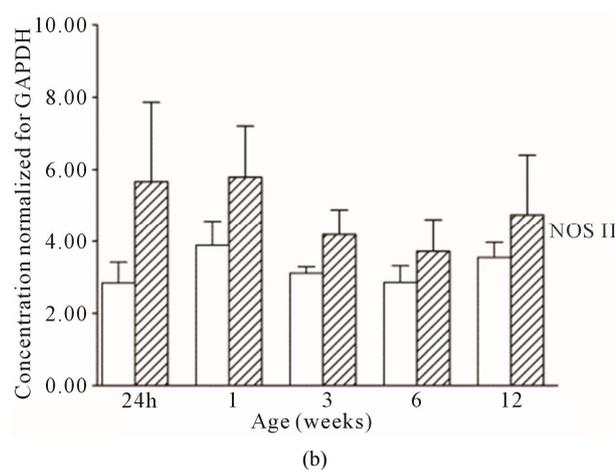
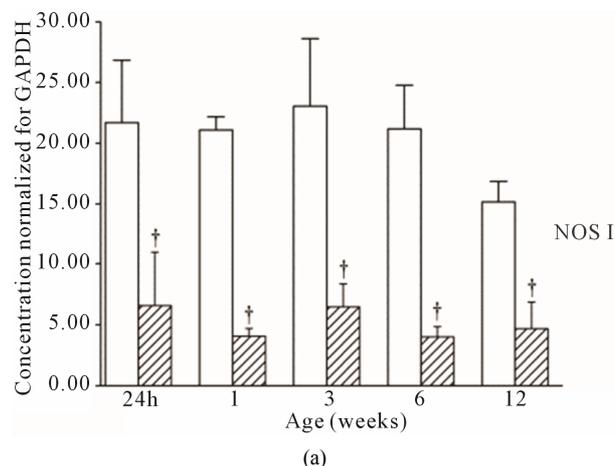
**Figure 2.** Representative RT-PCR images for each isoform NOS I, II, and III, the first three lanes are cortex (C) and the last three lanes are medulla (M).

were present in the cortex and medulla of the developing ovine kidney over the first three months of post natal life. NOS I protein levels were more than two-fold higher in the cortex as compared to the medulla at all age groups (**Figure 3(a)**). Cortical levels decreased from six to 12 weeks of age, whilst medullary levels remained low. NOS II protein levels were higher in the medulla than cortex during the first week post nately (**Figure 3(b)**). For the NOS III isoform, protein levels appeared predominant in the medulla as compared to the cortex at 24 h, and one and three weeks (**Figure 3(c)**). Immunoreactive levels of the three NOS isoforms, when corrected for the amount of protein loaded, showed similar results as when normalized for GAPDH.

### 3.3. Experimental Protocol III

All three NOS isoforms were localized within the ovine kidney post nately (see **Figure 4** and **Table 3**). NOS I transcripts were detected in highest abundance within the cortex from one day to three weeks. At 24 h, NOS I was localized to glomeruli, arterioles, and arteries. At one week, transcripts were still localized to glomeruli, and although fainter, the signal could be detected in arterioles. This trend continued at three weeks, with signal also present in proximal convoluted tubule (**Figure 4(G)**). Cortical signal for NOS I was negligible at six to 12 weeks. NOS II showed the lowest abundance overall, with transcripts negligible after six weeks (**Figure 4(K)**). At day one, NOS II transcripts appeared to be present in glomerular arterioles and *macula densa*, and at one week, NOS II was predominantly localized to glomeruli, although some faint staining could be detected in tubular cells.

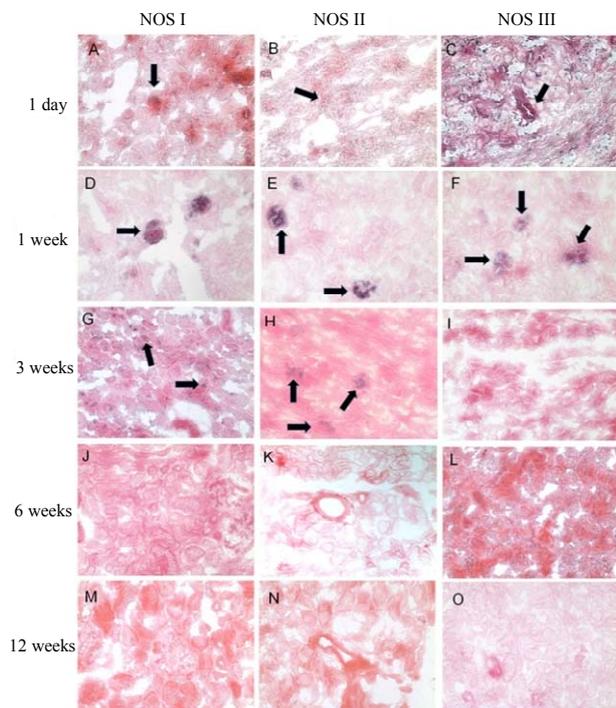
Staining was still positive in glomeruli at three weeks but not detectable at six and twelve weeks. NOS III



**Figure 3.** NOS protein expression cortex, open bars; medulla hatched bars. † $p < 0.05$  compared to cortex.

showed predominant transcription within the mesangium of the glomeruli and arterioles at 24 h, and increased within the medulla as compared to the cortex after three weeks; peritubular capillaries were devoid of signal (**Figures 4(C)** and **(F)**).

With respect to overall scores (**Table 3**), NOS I transcripts showed a down regulation in signal with post



**Figure 4.** *In situ* hybridization of NOS isoforms. Upper panel to lower panel: 24 h, one week, three weeks, six weeks, twelve weeks. Left column to right column: NOS I - III. All pictures show ovine cortical renal tissue treated with anti-sense digoxigenin coupled riboprobes at 200 $\times$  magnification. Localization to specific structures is shown by arrows.

natal age, with a substantial decrease after three weeks. The medullary score, which was highest within the one day age group, was less than that of the cortical score at all ages. The NOS II isoform had the lowest scores overall of all three isoforms. There was again, a substantial decrease of NOS II after three weeks. Within the first three age groups the cortical signal was substantially higher than that of the medulla. NOS III showed low to moderate signal at all age groups. There was a slight decrease in cortical signal at three, six, and twelve weeks.

#### 4. DISCUSSION

In the present experiments, we measured mRNA and immunoreactive protein expression for NOS I, II and III from day one to 12 weeks of post natal life in the ovine kidney and localized transcripts intra-renally. Novel findings of our experiments are that all three NOS isoforms are present within the developing ovine kidney from the day of birth, with profiles of mRNA expression and localization being developmentally regulated. This study provides the first evaluation of all NOS isoforms concurrently within the developing ovine kidney and a detailed time-line of their distribution from the day of birth through to three months. These data may help to explain effects of NO in modulating renal hemodynamics

and function early in life.

Kidney tissue was evaluated from day one, where nephrogenesis is complete near term in sheep, through to juvenile young adult sheep at pubescence (12 weeks). This profile provides a thorough range of ages from the time of birth through the transition of the newborn period (over the first three weeks) and to the young adult stage (at 12 weeks). All three NOS transcripts were localized to various extents within glomeruli at one week. Interestingly, all cortical NOS enzyme transcripts decreased by 12 weeks of post natal age, with changes occurring over the period three to six weeks. The increased abundance of all three NOS transcripts within the cortex in the immediate newborn period and the decreases detected postnatally provide evidence to support the premise that basal levels of NO may regulate glomerular function to a greater extent early in life (perhaps through all three isoforms), thus supporting our *in vivo* findings. This was especially evident for NOS I, in that mRNA as well as protein expression were considerably greater in cortex than medulla at all postnatal ages with cortical expression decreasing as postnatal maturation proceeded. In young adult (~3 months) and senescent Fisher rats (~24 months), NOS I and NOS III transcripts are opposite—that is, levels are greater in medulla as compared to cortex [18].

Localization of NOS II transcripts to the glomerulus at one week may also have implications for this enzyme in regulating GFR, a previously unidentified physiological role for the so-called inducible isoform. Physiological studies are, however, likely warranted before any definitive conclusions can be made regarding the function of the NOS II isoform in influencing kidney function in the immediate newborn period. Lack of statistical significance for the profile of changes in protein expression likely reflects the small N's studied, and the robust tests applied. However, the trends for protein expression, in general, paralleled mRNA expression for all isoforms. The current data also illustrate a general decrease in all three NOS isoforms by ~six weeks of age, but no specific increase in NOS expression or localization in or around tubular structures which might be expected if there were alterations in tubular reabsorption of electrolytes by NO with age. Therefore, it is more likely that the tubular effects of L-NAME treatment observed *in vivo* at six weeks result from changes in other factors which directly modulate tubular function.

NOS isoforms are widely expressed in the kidney and changes in their phosphorylation state govern NO production. For example, there are known serine/threonine phosphorylation sites on NOS I and NOS III. Dephosphorylation of these sites results in increased NOS activity and NO production. In the present study, all three isoforms (including NOS II) were present in the

**Table 3.** *In situ* hybridization scoring.

	24 h		1 week		3 weeks		6 weeks		12 weeks	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla
nNOS	+++	++	++	+	+++	++	+	+	+	+
iNOS	++	+	+	++	++	+	-	-	+	-
eNOS	++	++	++	++	+	++	+	+	+	++

Data represent averages of scoring as absent (-), low (+), moderate (++) and high (+++) signals.

immediate newborn period demonstrating the importance of the NO pathway in the adaptations of the kidney to post natal life.

Interestingly, the spatial distribution of the three NOS isoforms intrarenally overlapped in glomeruli and arterioles at some stages of post natal development perhaps providing evidence of redundancy of the system during the early newborn period. In the adult kidney, an interaction between the various NOS isoforms in influencing renal hemodynamics and function has been well characterized and at least the constitutive isoforms are known to exert specific functions under physiological conditions, exhibiting distinct localization and regulation [19-28]. For example, constitutive expression of NOS I mRNA is primarily in the *macula densa* and postganglionic nerve fibres [21,29-31] and implicated in the control of glomerular ultrafiltration and tubuloglomerular feedback [21]. NOS I has also been detected in medullary interstitial cells, and in principal cells of the collecting duct [32,33], although its role(s) in influencing water or electrolyte transport at these sites *in vivo* is not well understood. NOS III is predominantly expressed in vascular endothelial cells, hence its importance in regulating vascular resistance, tissue perfusion, oxygen consumption, as well as glomerular capillary pressure [20,26,34]. The constitutively expressed NOS III isoform is also reported to modulate tubulo-glomerular feedback, medullary blood flow, and NO-mediated inhibition of Cl<sup>-</sup> transport in the medullary thick ascending limb [35].

Consistent with our current findings, intra-renal distribution of all three NOS isoforms has been shown by Holmqvist *et al.* (2005) [36] in the mouse kidney, with NOS I localized predominantly to *macula densa* cells and NOS III restricted to vascular endothelial cells of glomerular capillaries, and small and large arteries. Their results also showed a down regulation of the constitutive isoforms (NOS I and III) and an up regulation of the inducible isoform (NOS II) after lipopolysaccharide treatment [36] Therefore, all intrarenal NOS isoforms may participate locally in different physiological processes both within the adult kidney and, in the newborn, developing kidney.

Generally considered an inducible isoform, NOS II is tonically expressed along the rat nephron including pre-

dominantly cells in the proximal straight tubule, thick ascending limb, distal convoluted tubule and inner medullary collecting duct suggesting its role in regulating tubular function [37,38]. NOS II can also be regulated via transcriptional and post-transcriptional mechanism by bacterial lipopolysaccharides, cytokines, and other inflammatory mediators; whether NOS II plays any significant regulatory roles under physiological conditions remains to be determined [19-20,39].

Changes in expression of NOS isoforms in the present study appeared to be most marked after three to six weeks, although there were slight discrepancies with regard to the timing of these changes between the methods employed to evaluate NOS mRNA expression—RT-PCR and *in situ* hybridization, which can be reconciled by their differences in signal detection. There was a general consistency between *in situ* and RT-PCR results where NOS I transcripts showed a down regulation in signal with post natal age, with a substantial decrease after three weeks. There was again a substantial decrease of NOS II after three weeks. The NOS III trends were consistent with RT-PCR findings although the cortical signal began to decrease earlier in the *in situ* hybridization studies as compared to RT-PCR results. Protein expression measurements similarly were not uniformly parallel with mRNA expression measurements, though again this is not surprising based on similar observations in ovine fetal lung tissue by Parker *et al.* (2000) [40], ovine fetal adrenal gland by Monau *et al.* (2009) [41], and rabbit placenta by Kahn *et al.* (2011) [42]. Whilst the underlying mechanisms of the changes occurring in NOS mRNA and protein expression at three to six weeks are not known at this time, it can be speculated that the marked changes in various aspects of kidney function across this time-spectrum such as the developmentally regulated decrease in RVR, and increase in GFR and FF, could be associated with alterations in NO production intrarenally, perhaps through age dependent alterations in phosphoregulatory processes. If so, then, NO production may be one of the major players in influencing kidney function early in life, as in the adult.

NOS I and III mRNA gene expression has been demonstrated in piglets and adult pigs [6,7] with a greater expression of NOS I in immature as compared to mature

kidneys. In these studies, however, a detailed time-line demonstrating a developmental profile over which these changes occurred was not included. Fischer *et al.* [8] measured the expression and localization during post natal development of one NOS isoform (NOS I) and found that it follows a cortico-medullary pattern ranging from single cell expression in the immature nephron to its full presence in the *macula densa* of nephrons in the mature kidney; the strongest NOS I signals were seen in the distal tubule at day six, and in the afferent arteriole by day two of post natal life [8]. Also in the developing rat kidney, Hanm *et al.* [9] measured NOS III expression and intra-renal localization during fetal and early newborn life and showed a unique cortico-medullary pattern distinct from that observed in the adult with immunostaining greater in the developing cortex than medulla, and a strong expression of NOS III in the early stages of developing glomeruli and vasculature. To this literature, the current study provides measurements of cortical as well as medullary mRNA and protein expression and localizations of all three NOS transcripts intrarenally, over a three-month span, from the day of birth, throughout post natal development in the ovine kidney, and including the time periods over which physiological investigations into the role(s) of endogenously produced NO have also been carried out [2,3,5,43,44].

## 5. CONCLUSION

The present study provides the first concurrent evaluation of all NOS isoforms within the developing kidney, and a detailed time-line of intra-renal distribution over the first three months of post natal life. The results help to elucidate our previous observations of age-dependent physiological responses to removing endogenous NO by showing that there is a localization of NOS isoforms near or around the glomerulus early in the post natal period, supporting an important role for NO in influencing ultra-filtration during this critical time period. NOS isoforms decrease within the kidney as post natal maturation proceeds, providing evidence that the NO system—one of the most important intra-renal systems—is down regulated over a relatively short time span after birth, thus supporting a vital role for NO in the adaptation of the kidney to life after birth.

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