The Study of Nitric Oxide Synthase Expression, Function, and Regulation in the Renal Vasculature During Postnatal Renal Development

Brian Blake Ratliff
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THE STUDY OF NITRIC OXIDE SYNTHASE EXPRESSION, 
FUNCTION, AND REGULATION IN THE RENAL 
VASCULATURE DURING POSTNATAL RENAL DEVELOPMENT 

by 

Brian Blake Ratliff 
B.A. May 2000, Chowan College 

A Dissertation Submitted to the Faculty of 
Old Dominion University and Eastern Virginia 
Medical School in Partial Fulfillment of 
the Requirement for the Degree of 

DOCTOR OF PHILOSOPHY 

BIOMEDICAL SCIENCES (BIOLOGICAL CHEMISTRY) 

OLD DOMINION UNIVERSITY 
EASTERN VIRGINIA MEDICAL SCHOOL 
May 2006 

Approved by: 

Michael Solhaug, M.D. (Director) 
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ABSTRACT

THE STUDY OF NITRIC OXIDE SYNTHASE EXPRESSION, FUNCTION, AND REGULATION IN THE RENAL VASCULATURE DURING POSTNATAL RENAL DEVELOPMENT

Brian Blake Ratliff
Old Dominion University / Eastern Virginia Medical School, 2006
Director: Dr. Michael J. Solhaug

The newborn kidney is vulnerable to vasomotor acute renal failure (ARF) from adverse perinatal events or complications of prematurity. Nitric oxide (NO) vasodilation is vitally protective in this type of ARF, but its relationship with other vasoactive factors, such as angiotensin II (AII) has not been examined. In the immature kidney, nitric oxide synthase (NOS) isoforms, specifically eNOS and nNOS, are developmentally regulated, but their specific role and regulation are unknown.

The enhanced vasodilatory role of NO in the immature kidney was hypothesized to be attributed to regulatory, expressional, and functional differences in eNOS and nNOS isoforms from the adult. The objective of the dissertation was to: (1) determine which NOS isoform regulates immature renal hemodynamics by using functional whole animal studies utilizing intrarenal infusion of NOS inhibitors; (2) continue characterization of NOS expression in immature renal microstructures utilizing LCM (glomerular eNOS expression); (3) characterize expression and functional patterns of NOS isoforms, renin, and AT1 and AT2 receptors in immature preglomerular resistance microvessels utilizing novel microdissection techniques; and (4) determine AII regulation of NOS expression and function in the immature renal vasculature using AT1 and AT2 receptor inhibitors.
Isoform specific inhibition demonstrated nNOS is the major NOS isoform regulating neonatal, but not adult, renal hemodynamics. nNOS expression, greatest in the newborn’s preglomerular resistance microvessels, decreased, while eNOS expression increased, with maturation. NOS enzymatic activity was greater in the neonate’s preglomerular resistance microvessels, than in the adult’s. AT1 and AT2 receptor inhibition demonstrated AII regulation of neonatal NOS expression and function via both receptors. Newborn nNOS expression demonstrated enhanced sensitivity to AII receptor inhibition.

Dissertation conclusions include: nNOS is the major isoform regulating renal hemodynamics in the immature, but not the mature, kidney. Glomerular eNOS expression is developmentally regulated with differences between intracortical location of glomeruli. NOS enzymatic activity in preglomerular resistance microvessels is greater in the newborn, than in the adult, and may be due to upregulated nNOS expression. While AII regulates eNOS expression and NOS enzymatic activity, via AT1 and AT2 receptors, in newborn and adult preglomerular resistance microvessels, nNOS expression is regulated by AII only in the newborn.
This dissertation is dedicated to my parents, Forest and Judy, whose belief in me and whose personal strength has always inspired me, and the Lord, whose Light is my eternal Saviour.
ACKNOWLEDGMENTS

Many people have contributed to the successful completion of this dissertation. First, and foremost, without the tremendous opportunity granted to me by Dr. Michael Solhaug to work in his lab, and his continuing mentorship, this dissertation would never have been possible. Thank-you to Dr. KeWen Dong, Dr. Xiaoshan Liu, and Dr. Usa Kullprawithaya for training me, and providing me with the fundamental laboratory background required to get this dissertation started when I first arrived in Dr. Solhaug’s lab. Justin Rodebaugh, Miroslav Sekulic, and Stephanie Montgomery provided the assistance required for each experiment, thus allowing my overall research to be successful. For that, I will always be appreciative. Also, the success of my experiments would not of been possible without the assistance of the EVMS physiology department’s staff and resources. The use of Dr. Gerald Pepe’s and Dr. Russell Prewitt’s labs and equipment, and the unwavering helping hands of Dr. Buck Davies, Marcia Burch, and Brandy Dozier proved invaluable during research stage of this dissertation. A debt of gratitude also goes to Dr. Pleban for helping to get me started in the Biomedical Program. A special thanks goes to Dr. Solhaug, Dr. Pleban, Dr. Ali Khraibi, and Dr. Prewitt for taking the time to sit on my dissertation committee. The Jones Institute also assisted in making the experiments in this dissertation possible by providing access to vital equipment. Last, but not least, without the continued care and support by my family, Forest, Judy, and James, the inspiring love of my angel Christine, and the immeasurable blessings of the Lord, this dissertation would never be.
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CHAPTER I

INTRODUCTION

Understanding the mechanisms that produce the unique renal functions of the neonate following birth are of high physiologic and pathophysiologic relevance for clinical management of newborns. A variety of different factors combine to promote a renal functional state in the newborn that is different from that of the adult. The newborn mammal, including humans, exhibits lower renal blood flow (RBF) higher renal vasculature resistance (RVR) lower glomerular filtration rate (GFR) and higher reabsorption of sodium as compared to the adult counterpart. The low RBF in conjunction with low GFR are produced by a high RVR. The uniqueness of the neonate’s renal hemodynamics impacts newborn clinical management, and predisposes the immature kidney to develop vasomotor-mediated acute renal failure (ARF) due to a variety of perinatal pathologies such as hypoxia, ischemia, shock, and prematurity complications.

The neonate’s unique renal condition is sustained by a crucial balance between vasoconstrictors, such as All, and the counter-acting vasodilator nitric oxide (NO). Angiotensin II (All) is highly activated at birth and maintains the high RVR found in the newborn kidney. A balance between these vasoactive factors in the developing kidney is the paramount mechanism that modulates renal hemodynamics during the postnatal period of development by influencing RVR reduction, with subsequent increases in RBF and GFR.

This dissertation follows the style of the American Journal of Physiology Regulatory Integrative Comparative Physiology.
NO plays a greater role in modulating renal neonatal hemodynamics under physiologic and pathophysiologic conditions. Current research suggest NO is the most important counter regulator of vasoactive constrictors in the developing kidney. This could be due to the developmental expression pattern of the NO-synthesizing enzyme nitric oxide synthase (NOS). Previous studies have identified two nitric oxide synthase isoforms, both of which are developmentally regulated in the immature kidney, endothelial NOS (eNOS or NOS III) and neuronal NOS (nNOS or NOS I) (131, 132). The enhanced participation of NO in the immature kidney is attributed to the regulated difference in expression of nNOS and eNOS in the neonate and during postnatal renal maturation as compared to the adult. Previous studies on NOS isoform expression in the immature kidney have found that both nNOS and eNOS mRNA and protein levels undergo distinct and differing whole kidney and corticomedullary developmental regulation (128, 129).

While studies have shown the developmental patterns of the NOS isoforms in the whole kidney and cortex/medulla regions, the distribution, expressional abundance, and the functional capabilities of each isoform in specific segments of the developing renal vasculature have not been reported. The changes in renal hemodynamics that occurs during development are modulated primarily in the glomerular resistance vessels, including the afferent, interlobular, and efferent arterioles. The maturational patterns of NOS isoform expression and NO function in the developing glomerular resistance vessels are unknown.
The role of NOS isoforms and the production of the vasodilator NO appear to be essential to keep in balance the enhanced vasoconstriction found in the newborn. In order to further understand the importance of the enhanced role of NO in the immature kidney, the distribution, expression and function of the NOS isoforms in these specific renal arterioles must be examined. Insight into the characteristics mediating the neonate’s renal hemodynamic state may be reflected in ARF prevention and treatment.

Several characteristics of nNOS in postnatal renal maturation suggest that this isoform may importantly participate in renal hemodynamics after birth. Whole kidney nNOS gene and protein expression are greater in the developing piglet kidney than the adult throughout postnatal renal maturation. nNOS also demonstrates an interdependent morphological relationship with the RAS. nNOS in the postnatal kidney may have interaction with the RAS.

The mechanisms that regulate NOS expression in the developing renal vasculatory system have not yet been identified. Preliminary studies have indicated that AII stimulates increases in whole kidney nNOS and eNOS expression in the kidney via AT1 receptors as seen in AT1 receptor inhibitory experiments. The stimulation of nNOS and eNOS by AII via the AT1 receptor has been linked to increases in cytosolic calcium. However, whole kidney experiment models do not delineate the specific regulatory mechanisms that are utilized by discrete renal vascular segments during development.

The enhanced vasodilatory role of NO in the immature kidney was hypothesized to be attributed to regulatory, expressional, and functional differences in eNOS and nNOS isoforms from the adult. The objective of the dissertation was to: (1) determine
which NOS isoform regulates immature renal hemodynamics by using functional whole animal studies utilizing intrarenal infusion of NOS inhibitors; (2) continue characterization of NOS expression in immature renal microstructures utilizing LCM (glomerular eNOS expression); (3) characterize expression and functional patterns of NOS isoforms, renin, and AT1 and AT2 receptors in the immature preglomerular resistance vasculature utilizing novel microdissection techniques; and (4) determine All regulation of NOS expression and function in the immature renal vasculature using AT1 and AT2 receptor inhibitors.
CHAPTER II

BACKGROUND

NOS IN THE KIDNEY

General description of NO

The vasorelaxative properties of nitric oxide (NO) were first discovered in 1979 by Gruetter et al. (73). However, it wasn't until 1987 that Ignarro et al. and Palmer et al. identified endothelium-derived relaxing factor (EDRF) as NO, thus realizing mammalian cells produce endogenous NO (73). NO is a lipophilic gas with unique physiological properties. NO is synthesized from the single amino acid precursor L-arginine by the enzyme nitric oxide synthase (NOS) with citrulline as a by-product. The NOS enzyme requires a variety of cofactors in order to generate NO from L-arginine including NADPH, calmodulin, FAD, FAM, heme and BH₄. In the early 1990’s, three different nitric oxide isoforms were cloned and characterized for the first time thus defining the biological source of nitric oxide in mammals. The three NOS isoforms identified are: neuronal NOS (nNOS), inducible NOS, (iNOS), and endothelial NOS (eNOS). Alternatively, the isoforms are numerically identified: NOS I or NOS 1 (nNOS), NOS II or NOS 2 (iNOS), and NOS III or NOS 3 (eNOS). The text here will refer to the NOS isoforms as nNOS and eNOS. Once produced, NO, acting in either an autocrine or paracrine fashion, can diffuse across cell membranes and interact with guanylate cyclase within vascular smooth muscle cells and influence calcium flux,
contractile protein sensitivity, and inhibit phosphodiesterase activity thus reducing cAMP degradation resulting in vasodilation of renal arterioles.

All three NOS isoforms are present in the adult kidney (9, 92). Two isoforms, nNOS and eNOS, are constitutively expressed and are calcium dependent within the immature kidney. iNOS is present only in low basal abundance in both the adult and immature kidney (128), not located in the renal resistance arterioles (92), and does not play a role in adult physiologic renal hemodynamic function (92). Furthermore, iNOS requires a number of activators under pathophysiological conditions that include lipopolysaccharide, interferons, and other cytokines (126).

In the adult rat kidney, both eNOS and nNOS are located in renal resistance arterioles and demonstrate enzymatic activity in these vessels (92, 150). nNOS may also alter renal hemodynamics from its location in the adult rat macula densa (9, 149). Newer studies also suggest that NO may mediate adjacent cellular function in the macula densa without direct diffusion (111). Other studies have indicated that NO derived from nNOS, possibly from perivascular nerves at neuromuscular junctions and/or in vascular smooth muscle cells, can effect adult microvessel vasoactivity (76). NO derived from eNOS located in the endothelium of microvessels has been proven repeatedly to contribute to adult microvascular vasoactivity. However, little is known about NO production or the localization and expression of eNOS and nNOS in the immature postnatal kidney, particular in the preglomerular resistance vessels. The exact role of eNOS and nNOS in the immature kidney’s renal hemodynamics and the role each isoform plays in modulating AII vasoconstriction is unknown.
NO/NOS in postnatal renal hemodynamics

The newborn mammal, including humans, exhibits lower RBF, higher RVR, and lower GFR than the adult counterpart (95, 151). The postnatal maturation of renal hemodynamics involves synchronous decreases in RVR accompanied by increases in RBF and GFR to adult capability (5, 51, 64). The major factor influencing the maturational increase in RBF and GFR is the progressive decrease in RVR (51, 79).

RVR in the developing kidney is influenced by structural factors, the number of existing vascular channels, as well as functional factors, the arteriolar resistance offered by each channel (6, 30). Several studies confirm that in the developing kidney, the functional maintenance of vascular tone, principally through a balance of vasoactive factors, is the paramount mechanism of the modulation of renal hemodynamics (5, 71, 126). The maturational changes in RBF and RVR must proceed normally to achieve adult capability for GFR (3, 151) and sodium homeostasis (130).

The primary vasoactive substance that causes the newborn’s elevated RVR is the RAS. All components of the RAS are highly expressed in the developing kidney. Many studies have indicated that an intact and functional RAS are required for normal kidney development. The highly activated All is the major vasoconstricting factor that maintains the high RVR, whereas NO is the most important vasodilator counteracting All, and other vasoconstrictors, such as endothelin (14, 52, 120). Fetal angiotensin levels are twice that found in the maternal plasma and circulating levels of All remain high during the newborn period and decrease with age (36). All elicits its effect on the porcine vasculature through two subtypes of receptors, the AT1 and AT2 receptors. It is believed the AT1 receptor elicits vasoconstrictor and hypertrophic effects on the
vasculature while the AT2 receptor mediates vasodilation and possibly proliferation. The intrarenal infusion of a non-peptide-specific AT1 receptor antagonist produces greater increases in renal blood flow in newborn piglets than in adults, a finding that further suggests All is a more important renal vasoconstrictor in the developing piglet than in the adult pig (132). However, the full functions of the two All receptors have yet to be fully identified in the immature piglet kidney.

Understanding the mechanisms that produce the unique neonatal renal functional state has physiologic and pathophysiologic relevance for the clinical management of the newborn. The newborn's unique renal hemodynamic state impacts neonatal clinical management, and predisposes the immature kidney to develop vasomotor-mediated ARF, caused by a variety of perinatal pathologies. Neonatal renal hemodynamics (3, 64, 151), contribute to the newborn's altered pharmacokinetics of renally excreted medications, and avid tubular sodium reabsorption, which blunts the expected natriuresis of an acute saline load (130). The newborn's renal condition also importantly participates in renal pathophysiologic conditions, such as ARF due to relatively common occurring adverse perinatal events, including hypoxia, shock, and complications of prematurity (6).

Gaining an understanding of the role of NO and the factors regulating NO function in the immature kidney may provide insights into creating therapies directed at the prevention or treatment of ARF. The maturation of renal hemodynamics is pivotal to the achievement of fully integrated renal-cardiovascular homeostasis. Investigating the role of NO in this maturational process may reveal causes of disrupted renal-
cardiovascular function in the adult leading to pathologic conditions such as hypertension.

**NOS in the adult kidney**

NO plays a major role in the maintenance of RBF and GFR in a number of adult animal models in a variety of experimental conditions (42, 83). Both systemic and intrarenal administration of competitive NOS inhibitors, such as L-NAME (123), leads to decreases in RBF and, depending on the method, reduction in GFR (12, 42, 48).

In the adult kidney, nNOS localizes predominantly to the macula densa, Fig. 1, with additional expression in the resistance arterioles, Bowman's capsule, thick ascending limb and collecting duct (7, 8, 81, 92, 138). nNOS mRNA has been identified in microdissected renal vascular segments including the afferent arteriole (92). NO produced from nNOS participates in regulating the glomerular microcirculation (9, 32, 62) and glomerular filtration rate (123). Studies suggest that nNOS-derived NO

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**Fig. 1.** NOS localization in the adult kidney.
participates in TGF regulation of preglomerular (afferent arteriole) vascular tone (63, 67, 147, 148). The exact mechanism of macula densa nNOS regulation of afferent arteriolar tone is not known. Rather than directly diffusing into the afferent arteriole, NO from nNOS, acting within the macula densa, stimulates a cGMP-dependent signaling pathway producing vasodilation (111).

Both eNOS mRNA and protein are found in the adult afferent and efferent arterioles (81), (Fig. 1). The direct study of eNOS-derived NO on the glomerular microcirculation is difficult, because of the lack of an eNOS-specific inhibitor. Most studies suggest that the local effect of eNOS may be to function as an autoregulatory factor on the afferent arteriole (15, 42, 83). eNOS-derived NO, produced locally in the glomerular microcirculation, modulates RBF and counter acts the effects of AII (42).

NOS in the postnatal developing kidney.

Several reports identify NO as a vital participant in postnatal developing renal hemodynamics, functioning as a critical vasodilator under physiologic and pathophysiologic conditions (11, 105, 124, 126). The immature renal vasculature is highly responsive to intrarenal NO stimulation with acetylcholine and NO inhibition with L-NAME (126). Utilizing L-NAME in a variety of animal models including anesthetized piglets (119, 124, 125), conscious lambs (121), and isolated perfused rabbit kidneys (125), support an enhanced functional role for NO in the immature kidney, critically maintaining RBF and GFR under physiologic conditions. Intrarenal infusion of the NOS inhibitor, L-NAME, in both whole animal (piglets) (131) and isolated perfused kidneys (rabbits) (124), produces greater renal hemodynamic responses in the
immature kidney than in the adult. Fig. 2 demonstrates the intrarenal infusion of L-NAME in immature piglets and adults (131). At 3 μg/kg/min, L-NAME produces greater responses in RBF and RVR in the piglet than in the adult. Non-specific NOS inhibition results in increased preglomerular resistance at the afferent arteriole, reduction in glomerular ultrafiltration coefficient, and variable effect on the postglomerular efferent arteriole (42, 83). Intrarenal perfusion of the NOS inhibitor L-NAME caused greater changes in RVR, RBF and GFR in the newborn piglet than in the adult (121, 127, 131). Pre-perfusion with an AT1 receptor inhibitor prior to L-NAME renal perfusion blunted the effects of L-NAME in the newborn kidney, but not in the adult (42). These studies indicated that not only was All a more important vasoconstrictor in the newborn kidney, but also that NO is a more important regulator of renal function in the newborn through modulation of the RAS (42). The enhanced role of NO in the neonate kidney is further supported by the distinct developmental patterns in the postnatal maturing porcine kidney.

NO is also a critical vasodilator during neonatal pathophysiologic conditions, particularly in conditions of perinatal hypoxemia, which is associated with respiratory or cardiovascular stress. These conditions induce an intense renal vasoconstriction, mediated by All, resulting in vasomotor ARF (6). In newborn rabbits, the renal hemodynamic responses to L-NAME are significantly greater during hypoxemia (11). NO protects the immature kidney from the deleterious effects of adverse perinatal events. The loss of NO protection renders the newborn kidney vulnerable to increased
constriction by highly activated vasoconstricting factors such as AII, and possibly endothelin (139). The immature kidney, therefore, requires an intact NO system to prevent vasomotor ARF.

Increased NO vasoactivity in the developing kidney may be due to a difference in localization and expression of NOS compared to the adult. The recently published postnatal pattern of steady state quantification by RNase protection assay (RPA) of whole kidney nNOS mRNA is shown in Fig. 3 (used with permission from APS) (128). Every age studied up to 21 days, has significantly greater nNOS mRNA than the adult. The newborn kidney demonstrates the greatest nNOS mRNA gene expression. With increasing age, nNOS mRNA gene expression progressively declines. The lowest level occurs in the adult. Whole kidney nNOS protein content by Western Blot, not shown, (128) parallels the mRNA pattern almost identically. In Fig. 4 (used with permission from APS), cortical and medullary nNOS protein content also demonstrates distinct developmental patterns during postnatal renal maturation.
Fig. 3. The mRNA expression of whole kidney nNOS. *P < 0.05, newborn and 7 days old vs. adult. 

Fig. 4. Corticomedullary nNOS expression in the kidney. Statistical significance not acheivable. 
Medullary nNOS protein content is proportionately greater than cortical at all ages studied; newborn, 14 day old piglets, and adults. Medullary nNOS, greatest in the newborn, decreases with age in a pattern that parallels whole kidney nNOS to the lowest level in the adult. Cortical nNOS remains at the same level from newborn to 14 days of age, respectively, then declines to the adult level.

The expression of eNOS during renal maturation has also been recently published (129). Experiments measured whole kidney mRNA gene expression by RT-PCR and protein content by western blot, and corticomedullary protein content by Western Blot. Additionally, whole kidney eNOS mRNA expression from the same kidney sample for nNOS and eNOS was determined. The latter experiments were designed to validate the expression patterns obtained from kidney samples that came from separate animals. As shown in Fig. 5 (used with permission from APS), the pattern of whole kidney eNOS mRNA expression differs from nNOS (Fig. 3). In the developing kidney eNOS is higher in the newborn kidney and dramatically decreases to the lowest level at day 7. Then, the expression level of eNOS significantly increases, and is maintained at this level throughout the maturation until adulthood. Whole kidney eNOS protein content, not shown, (119), has a similar pattern to that of mRNA expression. The high eNOS protein content in the newborn decreases to the lowest level at day 7, then increases by day 14 and remains at this level until adulthood.

eNOS protein, Fig. 6 (used with permission from APS), also demonstrates a pattern that differs form nNOS. Cortical eNOS protein was highest in the newborn. Cortical eNOS protein was greater than medullary in newborn and 7 day piglets. The adult has relatively equivalent cortico-medullary eNOS protein expression.
Fig. 5. The mRNA expression of whole kidney eNOS. *P < 0.05, 7 days old vs. newborn and adult. *Am J Physiol Regul Integr Comp Physiol 280: R1269-R1275, 2001.*

Fig. 6. Corticomedullary eNOS protein expression in the kidney. *P < 0.05, newborn vs. all ages. &P < 0.05, 7 days vs. all ages. #P < 0.05, adult vs. all ages. *Am J Physiol Regul Integr Comp Physiol 280: R1269-R1275, 2001.*
The postnatal whole kidney nNOS and eNOS experiments are the first to demonstrate the postnatal renal expression of NOS isoforms throughout the maturational spectrum to adulthood. When compared, both the nNOS and eNOS isoforms undergo distinctively different developmental expression patterns, as seen in Fig. 7 (used with permission from APS). The nNOS and eNOS pattern support the hypothesis that developmental changes in NOS contribute to the differences in renal function between the immature and the adult. Both isoforms undergo developmental regulation. However, both isoforms have contrasting whole kidney and cortico-medullary patterns. These differing developmental patterns suggest nNOS and eNOS may also have differing functional contributions during postnatal renal maturation.

Now that whole kidney NOS mRNA expression and protein translation has been identified in the postnatal kidney, the next step in determining the role of NO/NOS in the neonate’s renal hemodynamics is to identify the specific localization and expression

Fig. 7. nNOS and eNOS mRNA expression in same kidney. #P < 0.05, 7 day vs. newborn and adult, *P < 0.05, newborn and 7 day vs. adult. Am J Physiol Regul Integr Comp Physiol 280: R1269-R1275, 2001.
of NOS isoforms in specific renal segments. nNOS is identified in the earliest stages of nephron morphogenesis, marking the location of the site of the macula densa throughout nephron formation. As the nephron becomes fully differentiated, but still maturing, nNOS increases in abundance, particularly in the thick ascending limb, and macula densa. With further maturation, as the nephron migrates centrally out of the superficial cortex closer to the juxtamedullary region, this tubular distribution recedes to the macula densa. In the adult, nNOS is mainly confined to the macula densa (85, 128).

The novel technique Laser Capture Microdissection (LCMD) was used by Solhaug et al. to acquire macula densas from porcine kidneys at ages newborn, 21 day and adult. Macula densas were analyzed for nNOS mRNA by RT-PCR (85). The nNOS mRNA expression in LCMD macula densas is illustrated in Fig. 8., which graphs the results of the quantification of LCMD macula densas for the three age groups. Using cyclophilin as an internal control, nNOS mRNA is greatest in the newborn compared to the two other age groups. Macula densa nNOS mRNA, lower in

![Bar graph](image_url)

*Fig. 8. Quantification of nNOS mRNA in LCMD macula densas (unpublished). N=8.*

*P < 0.05, newborn and 21 day vs adult, #P < 0.05, newborn vs. 21 day.*

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the 21 day-old piglet than in the newborn, appears to decrease with advancing age in the
developing kidney. The quantity of this isoform in the macula densa at 21 days of age,
when nephrogenesis is complete, is greater than in the adult. The lowest level of macula
densa nNOS mRNA is seen in the adult.

In summary, both eNOS and nNOS undergo significant regulation after birth in
the immature kidney. Studies show distinct, but differing patterns in whole kidney,
cortico-medullary, as well as selected components of the renal microstructure including
the macula densa. These studies support the hypothesis that the enhanced role for NO in
the immature kidney is due to quantifiable differences in the NOS isoforms from the
adult kidney. However, the developmental pattern of NOS isoforms in distinct renal
segments, such as the renal resistance vasculature and glomeruli, are not known.

NOS IN THE RESISTANCE ARTERIOLES

NOS in the adult renal resistance arterioles

In the adult, acute systemic and intrarenal NOS inhibition increased vascular
resistance in both the afferent and efferent arterioles (5). Nonspecific NOS inhibition
results in increased preglomerular resistance at the afferent arteriole, decrease in
glomerular ultrafiltration coefficient, and variable effects on the postglomerular efferent
arteriole (42, 83). Results of such studies indicate the importance of NOS derived NO in
maintaining normal adult renal hemodynamics. It is important to ask what isoform is the
source of the critical NO that modulates adult vascular renal hemodynamics. Many
studies have focused on eNOS as the major source of NO in the adult vasculature, while
NO derived from macula densa nNOS may effect vasoreactivity as well. However, localization and function of nNOS in the vasculature may also play a role in adult renal vasoreactivity.

In the adult kidney, increases in renal blood flow are associated with a decrease in resistance of all glomerular vessels, the interlobular, afferent, and efferent arterioles (4). The relationship between the lowered vasculature resistances of the renal resistance arterioles, characteristic of the adult kidney, and nNOS derived vasodilator NO is not fully understood. While the vast majority of studies have localized nNOS indiscriminately to either the cortex or the medulla, more specific immunohistochemistry and in situ hybridization experiments have localized nNOS to specific renal vasculature. Overall, contradictive results have arisen from studies that have further localized nNOS to segments of the renal vasculature. Some studies agree that nNOS is intrarenally distributed to the endothelium of afferent arterioles, efferent arterioles, the glomerular parietal epithelium, and the renal pelvic and perivascular nerves (81, 83, 126). In one serious of experiments on a variety of species including rat, mouse, guinea pig, rabbit, pig, and human kidneys, Bachmann et al. found glomerular arterioles showed stronger labeling of nNOS in the efferent than in the afferent endothelium, while at the capillary level, only the glomerular tuft showed NOS-positive endothelia (7). A subpopulation of renal nerves containing nNOS was found in perivascular connective tissue and near pelvic epithelium (7). Other studies have led to variable results indicating both the presence and absence of nNOS in the interlobular artery, afferent arterioles, and in the medullary vasa recta (3). The vast majority of studies agree nNOS is predominately localized to the macula densa, with further studies
implicating macula densa derived NO diffuses to the afferent arteriole where it participates in TGF regulation of preglomerular vascular tone (63, 67, 147, 148).

eNOS derived NO, produced locally in the glomerular microcirculation, modulates RBF and counter acts the effects of AII (42). Both eNOS mRNA and protein have been found in the endothelium of the glomerulus, arcuate artery, interlobular artery, afferent arteriole, glomerular capillaries, efferent arteriole, vasa recta bundles, and intrarenal arteries (81, 83, 126, 141). In one serious of experiments on a variety of species including rat, mouse, guinea pig, rabbit, pig, and human kidneys, Bachmann et al. found glomerular arterioles showed stronger labeling of eNOS in the efferent than in the afferent endothelium, while at the capillary level, only the glomerular tuft showed NOS-positive endothelia (7). The majority of functional eNOS in quiescent endothelial cells resides in caveola, the result of dual acylation. The NO produced by eNOS in endothelial cells of the resistance arterioles diffuses to adjacent smooth muscle cells and stimulates a cGMP-dependent signaling pathway producing vasodilation. Studies have demonstrated the importance of eNOS derived NO in counter balancing the effects of vasoconstrictors, such as AII, in the adult kidney. Deficiencies in the ability of eNOS to counter-act vasoconstrictors predispose individuals to pathophysiological conditions such as hypertension.

Studies by Patzak et al. (105) using adult mice demonstrated the functional role of NOS in the adult renal resistance arterioles. Perfused afferent arterioles exhibited a vasoconstrictive response to the nonselective NOS inhibitor L-NAME (105). The nNOS specific inhibitor L-SMTC was found to induce afferent arteriolar constriction in studies done with rats (131). However, the L-SMTC studies used whole nephron preparations
instead of dissected afferent arterioles to assess the effects of nNOS inhibition on arteriole diameter. Thus, inhibited NO production could be a result of macula densa nNOS inhibition instead of nNOS localized to the resistance arterioles. Overall, the data concerning functional roles of nNOS and eNOS in the resistance arterioles of the adult is very limited. The majority of studies to date have looked at the effects of NOS inhibition on whole kidney models. Thus, the functional contributions of nNOS and eNOS in the resistance arterioles and the subsequent effect on adult renal hemodynamics remains unclear.

NOS in the developing renal resistance arterioles

Initial increases in renal blood flow with maturation mainly results from the progressive reduction in RVR (51). The site of high renal vascular resistance in the newborn animal has been localized primarily to the preglomerular resistance vasculature, the interlobular artery and afferent arterioles (133). The decrease in renal vascular resistance with age may be modulated by developmental changes in the intrinsic properties of the renal resistance vasculature. However, very little is known about the myogenic and internal vasoactive capabilities of these vessels, or any developmental differences in resistance vessel responsiveness to vasoactive factors. Ultimately, the characteristics of postnatal renal hemodynamics is considered to be a balance between neurohormonal vasoactive factors. Both the vasoconstrictors and the vasodilators producing this immature renal condition have increased effects, intrarenal levels, or differing sites of action compared to the adult. Several studies have confirmed that in the developing kidney, the functional maintenance of vascular tone, principally
through a balance of vasoactive factors, is the paramount mechanism of the modulation of renal hemodynamics (5, 71, 126). The maturational changes in RBF and RVR must proceed normally to achieve adult capability for GFR (3, 13) and sodium homeostasis (130). The distribution and expressional patterns of NOS isoforms in the resistance arterioles of the neonate may play a role in kidney maturation, the decrease in RVR, and subsequent increases in RBF and GFR to adult efficiencies.

nNOS undergoes developmental expression patterns in whole kidney models. However, it is unknown if any such developmentally regulated nNOS pattern exists in the neonate's renal resistance vasculature. The expressional and distributional pattern, if any, to date has not been looked at in the resistance arterioles of the developing kidney. Fischer et al. (37) and latter localization experiments in piglets provided insight that vascular nNOS distribution and expression in the developing kidney may differ from that of the adult. However, the results of Fischer et al. (37) indicate the absence of nNOS in the resistance arterioles of the developing kidney, contradictive to other similar studies (126). Thus, such results mean either nNOS is not expressed in the resistance arterioles of the developing kidney of certain species, or that the sensitivity of experimental techniques used thus far may not allow for the identification of minute amounts nNOS mRNA in the resistance arterioles.

The enhanced expression of eNOS in the early stages of the developing kidney suggests eNOS may contribute to angiogenesis and/or critically participate in the renal hemodynamics of the immature kidney. Studies thus far have not obtained the entire picture regarding eNOS in the immature kidney. To date, studies have used only in situ hybridization and immunohistochemistry to identify eNOS localization in only a few
animal species. In the endothelial cells of the peritubular capillary, eNOS decreases in expression after birth. The endothelial cells of the arcuate artery strongly express eNOS from gestation throughout maturation into adult stages (53). After birth, eNOS expression in the vasa recta gradually increased in the renal medulla (53). Complete analysis of expression and distributional patterns of eNOS in the developing kidney, particularly in the renal resistance arterioles, has yet to be examined.

Information concerning function of NOS in the immature renal resistance arterioles has been confined to whole kidney studies. In whole kidney immature pig models, inhibition of NOS with either the nonselective L-NAME or the nNOS specific L-SMTC resulted in an increase in RVR and decreases in RBF and GFR (131). Whole kidney models fail to delineate the function of NOS isoforms in distinct renal resistance arterioles. Identification of specific functional contributions of NOS isoforms in the resistance arterioles would allow better understanding of the developing differences found in the immature kidney as compared to the mature kidney. Functional differences of NOS in the developing kidney may contribute to the maturation of neonatal renal hemodynamics to the level of adult proficiency. The function of NOS in the resistance arterioles of the neonate may play a role in the decrease of RVR, and subsequent increases in RBF and GFR, that occurs during development. NO appears to be required in the renal vasculature for normal physiological function during development, but the functional role of NO in the newborn renal vasculature remains incompletely studied. Understanding the function of NOS in the resistance arterioles during development may translate into improved prevention and treatment of neonatal ARF.
INTERACTION BETWEEN NOS AND AII IN THE KIDNEY

**AII stimulation of NOS in adult renal resistance arterioles.**

Thorup et al. was able to determine that AII stimulates NO release in isolated renal resistance arteries in experiments utilizing the measurement of NO production in isolated rat renal resistance arterioles (135). AII stimulation of NO was significantly attenuated by AT1 receptor blockade, thus suggesting the endothelium-dependent modulation of AII-induced vasoconstriction in renal resistance arteries is mediated, at least in part, by AT1 receptor-dependent NO release (135). In a separate set of experiments, Thorup’s findings were confirmed when it was determined that AII induced stimulation of NO release in isolated renal resistance arterioles is attenuated by simultaneous AT1 receptor blockade (136). The extent of NO production regulation by AII through the AT1 receptor in the resistance arterioles was expanded to the interlobular arterioles in latter experiments (57). AII produces vasoconstriction by a direct action on smooth muscle cells via AT1 receptors, as demonstrated in Figs. 15 and 17. These receptors are also present in the endothelium, but their function is poorly understood. Furthermore, AII has been found to increase intracellular free calcium in endothelial cells, which mediates vasoconstriction and possibly a counter vasodilatory affect. AII stimulated NO release from macrovascular endothelium may modulate the direct vasoconstrictor effect of AII on smooth muscle cells (109). To date, few experiments have attempted to identify the relationship between AII stimulation of NOS via the AT1 receptor in the resistance arterioles of adult models. There are presently no
studies that address the developmental pattern of AII stimulation of NOS via the AT1 receptor in the resistance arterioles of the developing kidney.

Vasoactive factors, such as AII, may play a role in the postnatal regulation of the renal NOS isoforms. In the postnatal developing kidney, the renin angiotensin system is highly activated at birth (45). Plasma AII exhibits a postnatal renal pattern that is similar to that of nNOS, greatest in the newborn, then decreasing with age (45, 107). Systemic and renal AII decreases with age in the postnatal period (45), including in maturing piglets (101, 132). Co-localization studies suggest an interactive relationship between nNOS containing cells and renin producing cells in the developing rat kidney (37). AII is considered to be the principle vasoconstrictor maintaining the high RVR, which sustains the low RBF and GFR, in the newborn and during maturation (119, 132). Previous studies have shown that AII is a more active vasoconstrictor in the immature piglet kidney. Acute intrarenal infusion of an AT1 antagonist altered renal hemodynamics in the immature kidney, but not in the adult, significantly increasing RBF in the piglet (132). The possibility that AII may be an important factor regulating NOS expression and function during postnatal maturation is supported by the following observations: AII may regulate NOS in the adult kidney, AII is highly activated at birth and undergoes postnatal changes similar to NOS, the vasoactive counter-modulating interrelationship between AII and NOS is more important in the immature kidney.

The abundance of AII receptors could impact NOS regulation during renal maturation. The AT receptor sub-types, AT1 and AT2, undergo postnatal changes in abundance. Previous studies have indicated the AT1 receptor, relatively low in the fetus, rises rapidly at birth, and remains relatively unchanged in abundance during
postnatal maturation (75, 140, 152). The AT2 receptor is more abundant in the fetus, drops rapidly after birth to very low levels that are maintained during postnatal maturation (152, 153). Thus, if the AT2 receptor participates in postnatal NOS regulation, it is in the immediate period after birth.

Of the two AT receptors, AT1 is more likely to mediate AII regulation of NOS. It is the predominant receptor shortly after birth, and remains so during maturation. Studies have demonstrated that AII vasoconstriction, mediated by the AT1 receptor, is more pronounced in the immature kidney (132). Based on these observations, our lab conducted a series of experiments to explore the regulation of NOS isoforms by AII focusing on the AT1 receptor in the postnatal maturing kidney. Acute AT1 intrarenal infusion pretreatment produced greater attenuation of the renal hemodynamic responses to L-NAME in the piglet compared to the adult. After pretreatment with acute intrarenal AT1 receptor inhibitor infusion, the percent changes in RBF and RVR responses to intrarenal L-NAME were attenuated to a greater degree in the immature kidney than in the adult. A similar functional interaction between NO and AII is seen in the isolated perfused kidney of the newborn rabbit (124). In the studies conducted by our lab, AT1 receptor inhibition (AT1X) significantly attenuated the renal hemodynamic responses to L-NAME in the immature kidney as compared to the adult. These findings in earlier studies utilizing acute intrarenal administration of an AT1 receptor antagonist confirm that, in the immature kidney, NO plays an enhanced vasodilator role, and demonstrate that AT1 inhibition modulates the responses to NOS inhibition to a greater degree in the neonate than in the adult.
Our lab was also able to determine whole kidney nNOS and eNOS mRNA gene expression following the unilateral intrarenal arterial infusion of an AT1 receptor antagonist, candesartan, at a dose that did not alter systemic blood pressure. Fig. 9 displays the effect of acute AT1X on the mRNA gene expression of the two NOS isoforms. Both nNOS and eNOS mRNA exhibit developmental patterns very close to those previously described (128, 129). In the newborn, acute AT1X profoundly decreased both nNOS and eNOS mRNA expression. Acute AT1X did not alter eNOS mRNA in the 7 day piglet or the adult. The 7 day old piglet kidney experienced decreased nNOS mRNA with acute AT1X. Interestingly, acute AT1X did not alter eNOS protein as measured by Western Blot (data not shown). However, nNOS protein expression was altered by acute AT1 receptor antagonist infusion. As seen in Fig. 10, acute AT1X decreased nNOS protein in the newborn, and possibly the 7 day old, but not the adult. These studies demonstrated that both nNOS and eNOS are upregulated by

![Graph showing mRNA expression of nNOS and eNOS](https://via.placeholder.com/150)

**Fig. 9.** Effect of acute AT1 receptor inhibition on nNOS and eNOS mRNA gene expression by RT-PCR (preliminary studies). N=3. Statistical significance not achievable.
All via the AT1 receptor in the immature kidney, most pronounced in the immediate newborn period. nNOS mRNA and protein are regulated in parallel in the newborn kidney, thus changes in this isoform would have a greater impact on NOS function. Although eNOS mRNA in the immature kidney was profoundly decreased, eNOS protein was not changed. This may be due to the length of time acute AT1X was administered, since chronic AT1X significantly alters eNOS protein expression. This discordant regulation of eNOS mRNA and protein is consistent with the findings of Hennington et al., who showed acute AII infusion in adult rats changed eNOS mRNA only, but chronic AII infusion also altered eNOS protein expression (58).

Fig. 10. Effect of acute AT1 receptor inhibition on nNOS protein by western blot (preliminary studies). N=3. POS = positive control, pig cerebellum, NEG = negative control, fetal bovine serum. Statistical significance not achievable.
MECHANISMS OF ALL REGULATION OF NOS VIA CALCIUM MOBILIZATION

Role of Calcium in Vascular Smooth Muscle Contraction and Relaxation.

Contraction of vascular smooth muscle (VSM) can be initiated by mechanical, electrical, and chemical stimuli. Passive stretching of VSM can cause contraction that originates from the smooth muscle itself and is therefore termed a myogenic response. Electrical depolarization of the VSM cell membrane will also elicit contraction, most likely by opening voltage dependent calcium channels (L-type and T-type calcium channels) and causing an increase in the intracellular concentration of calcium. Finally, a number of chemical stimuli such as norepinephrine, All, vasopressin, endothelin-1, and thromboxane A2 can elicit contraction. Each of these substances bind to specific receptors on the VSM cell (or to receptors on the endothelium adjacent to the VSM), which leads to contraction of the VSM. The mechanism of contraction can involve different signal transduction pathways all of which converge to increase intracellular calcium (77).

The mechanism by which an increase in intracellular calcium stimulates VSM contraction is illustrated in Fig. 11. An increase in free intracellular calcium can result from either increased flux of calcium into the cell through calcium channels or by release of calcium from internal stores (e.g., sarcoplasmic reticulum; SR). The free calcium binds to a special calcium binding protein called calmodulin. Calcium-calmodulin activates myosin light chain kinase (MLCK), an enzyme that is capable of phosphorylating myosin light chains (MLC) in the presence of ATP. Myosin light
Fig. 11. The mechanism by which an increase in intracellular calcium stimulates VSM contraction. See text for description.

Chains are 20-kD regulatory subunits found on the myosin heads. MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, smooth muscle contraction (77).

In addition to activating MLCK and thus contraction, the calcium-calmodulin complex activates NOS proteins. Binding of the Ca$^{2+}$/calmodulin complex to NOS is necessary for the enzyme’s catalytic activity to be functional. Substances such as acetylcholine, bradykinin, histamine, insulin, substance P, and All stimulate NO production through mechanisms involving Ca$^{2+}$/calmodulin complexes (Fig. 12). Increased flow velocity on the luminal surface of the vascular endothelium can also stimulate intracellular calcium release and thus increase NOS activity.

Calmodulin binds to both nNOS and eNOS only in the presence of micromolar concentrations of intracellular calcium (137). Binding of Ca$^{2+}$/calmodulin to its specific
Fig. 12. Illustration of the mechanisms involved in the production and subsequent diffusion of NO from endothelial cells into vascular smooth muscle cells, and the resulting relaxation of the vascular smooth muscle cells.

motif on NOS displaces an adjoining (117) inhibitory loop on both nNOS and eNOS, thereby activating the NADPH:cytochrome-c reductase activity promoting NADPH-dependent electron flow from the reductase domain to the oxygenase domain of the protein. NOS protein domains are illustrated in Fig. 13. Activation of NOS activity by Ca\(^{2+}\)/calmodulin is not affected by the absence or presence of heme and therefore seems to involve primarily the reductase region of the NOS enzymes (137). As intracellular calcium concentrations fall, calmodulin dissociates from NOS. Without bound Ca\(^{2+}\)/calmodulin, NOS does not catalyze citrulline formation and NADPH:cytochrome-
c reductase activity is barely detectable (137). This bimodal conceptualization of Ca\(^{2+}\)/calmodulin-dependent regulation of NOS has been greatly refined to include a complex array of protein-protein interactions.

![Diagram of NOS proteins and domains](image)

Fig. 13. The illustration of the domains of the NOS proteins.

All has been found to increase intracellular free calcium in endothelial cells, thus initiating vasoconstriction and possibly a counter vasodilatory affect. All stimulated NO release from macrovascular endothelium may modulate the direct vasoconstrictor effect of All on smooth muscle cells (109). When NO is formed by an endothelial cell, it readily diffuses out of the cell and into adjacent smooth muscle cells where it binds to a heme moiety on guanylyl cyclase and activates this enzyme to produce cGMP from GTP. Increased cGMP activates a kinase that subsequently leads to the inhibition of calcium influx into the smooth muscle cell and decreased calcium-calmodulin stimulation of myosin light chain kinase (MLCK). This in turn decreases the phosphorylation of myosin light chains, thereby decreasing smooth muscle tension.
development and causing vasodilation. There is also some evidence that increases in
cGMP can also lead to myosin light chain de-phosphorylation by activating a
phosphatase. The anti-platelet aggregatory effects of NO are also related to the increase
in cGMP (77).

Intracellular calcium concentrations, therefore, are very important in regulating
smooth muscle contraction. The concentration of intracellular calcium depends upon the
balance between the calcium that enters the cells, the calcium that is released by
intracellular storage sites (e.g., SR), and removal of calcium either back into storage
sites or out of the cell. Calcium is re-sequestered by the SR by an ATP-dependent
calcium pump. Calcium is removed from the cell to the external environment by either
an ATP-dependent calcium pump or by the sodium-calcium exchanger (77). Studies
have found that reduced sodium/calcium exchanger activity in the resistance arterioles
is present in hypertensive rats, thus indicating a role of calcium influx/efflux in
resistance arteriole constriction/dilation (28).

There are several signal transduction mechanisms that modulate intracellular
calcium concentration and therefore the state of vascular tone. Three different
mechanisms are described here: 1) phosphatidylinositol pathway, 2) G-protein-coupled
pathway, and 3) nitric oxide-cGMP pathway (77).

The phosphatidylinositol pathway (Fig. 14) in VSM cells is similar to that found
in the heart. Norepinephrine (NE) acting via alpha 1-adrenoceptors, angiotensin II (AII)
acting via All receptors, and endothelin-I (ET-1) acting through ETA receptors activate
phospholipase C (PL-C) causing the formation of inositol triphosphate (IP₃) from
phosphatidylinositol (PIP₂). The IP₃ then stimulates the sarcoplasmic reticulum (SR) to
release calcium. The formation of diacylglycerol (DAG) activates protein kinase C (PK-C), which can also contribute to VSM contraction via protein phosphorylation (77).

The G-protein coupled pathway (Fig. 14) either stimulates (via Gs protein) or inhibits (via Gi protein) adenylyl cyclase (AC), which catalyzes the formation of cAMP. In VSM, unlike the heart, an increase in cAMP (e.g., a beta-agonist such as epinephrine or isoproterenol) causes relaxation. Relaxation occurs as cAMP inhibits MLCK. This decreases MLC phosphorylation, thereby decreasing the interactions between actin and myosin. Therefore, drugs which increase cAMP (e.g., β2-adrenoceptor agonists, phosphodiesterase inhibitors) cause vasodilation (77).

A third mechanism that is very important in regulating VSM tone is the nitric oxide (NO)-cGMP system (Fig. 14). Increases in NO activate guanylyl cyclase causing increased formation of cGMP and vasodilation. The precise mechanisms by which
cGMP relaxes VSM is unclear; however, cGMP can activate a cGMP-dependent protein kinase, inhibit calcium entry into the VSM, activate K+ channels, and decrease IP₃.

The inducible form of NOS (iNOS, or NOS II) is not calcium-dependent, but rather is stimulated by the actions of cytokines (e.g., tumor necrosis factor, interleukins) and bacterial endotoxins (e.g., lipopolysaccharide). Induction of this enzyme occurs over several hours and results in a production of NO that may be more than a 1,000-fold greater than that produced by nNOS or eNOS. This is an important mechanism in the pathogenesis of inflammation (77). However, under normal physiological conditions, iNOS does not play a role in normal vascular smooth muscle relaxation, and thus, is not discussed here.

**Calcium channels in the resistance arterioles**

Alterations in membrane potential and voltage-mediated changes in L- and T-type calcium channel activity play a prominent role in smooth-muscle excitation-contraction coupling and are especially important for the regulation of tone in resistance arterioles (98). Gordienko et al. provided electrophysiological evidence for the presence of L-type voltage-gated calcium currents and also for T-type calcium currents in single vascular smooth muscle cells of the afferent arteriole (46, 55). Experiments using various calcium channel blockers and/or measurement of intracellular calcium concentration all agree that L- and T-type voltage channels, plus additional calcium release from internal stores, regulate intracellular calcium concentrations in the preglomerular resistance arterioles (4, 27, 33, 34, 36, 56, 91, 102). The presence of T- and L-type channels in the preglomerular resistance arterioles was confirmed by
Hansen et al. using mRNA expression studies (55). Furthermore, Hansen et al. identified the expression of P-/Q-type voltage-gated calcium channels in renal afferent arterioles (44). Studies on young rats by Arendshorst et al. have found that store-operated calcium entry is exaggerated in preglomerular VSM cells (35,118).

Recent studies have determined that although the overall responses are similar, the calcium signaling pathways differ appreciably between pre- and postglomerular resistance vessels (4). The current theory is that calcium entry through voltage-operated channel predominates in preglomerular arteries/arterioles and that either calcium mobilization and/or calcium entry through channels insensitive to voltage are primary in efferent arterioles. Various studies have shown AII induced afferent vasoconstriction is closely coupled to membrane depolarization (90). In the afferent arteriole, AII causes VSM cells to depolarize, reducing the membrane potential from -40 to -29 compared with an unchanged resting potential during stimulation of the efferent arteriole (89). The stimulation by AII in the afferent arteriole is mediated predominately by opening of voltage-gated L-type calcium entry channels as shown using microdissected isolated afferent arterioles and individual renal VSMC (21,22,28,65,69). Iversen et al. was able to prove the presence of AII stimulated calcium entry via voltage-gated calcium channels in the interlobular arteriole of the rat (68). AII has also been found to stimulate calcium release in preglomerular smooth muscle cells from the internal calcium store, including the sarcoplasmic reticulum (110).

Studies aimed at identifying calcium channels in postglomerular resistance arterioles have provided conflicting results. Different studies have found both the absence and presence of T- and L-type channels in the efferent arterioles (4,36,56,
Perhaps an explanation for such discrepancy can be attributed to experimental variations between groups, in particular, differences in the regions of the kidney that were used for experimentation. The mRNA expressional studies by Hansen et al. concluded that voltage-gated calcium channels are expressed and of functional significance in juxtamedullary efferent arterioles, and in outer medullary vasa recta, but not in cortical efferent arterioles (55). Other studies have found the internal calcium stores in the VSM cells of the efferent arteriole have an enhanced role in modulating internal calcium concentrations, a role that is more influential on calcium flux inside the cell as compared to that of L-/T- type voltage channels (27, 91). Collectively, results to date suggest that AII action on the efferent arteriole is probably due to calcium mobilization from internal stores and/or calcium entry through dihydropyridine insensitive nonspecific cation channels (4, 96). The latter may reflect calcium release-induced calcium entry via voltage-independent channels (4). Fig. 15 illustrates several signaling pathways by which AII can elicit changes in cytosolic calcium concentration and contraction.

Although AII preferentially constricts the efferent arteriole, it is the afferent arteriole that has attracted attention in the interaction between AII and NO. This is mainly because it is considered likely that NO is the main vasodilator of the afferent arteriole. It has yet to be determined if the intracellular calcium mobilization elicited by AII regulates NOS expression and function via the T- / L- type and/or internal store calcium channels in the kidney. Furthermore, the mechanism by which AII regulates NOS in the resistance arterioles has not been examined in the developing kidney.
Extracellular

Voltage-Operated 
L-Type Ca\(^{2+}\) Channel

Voltage-Independent 
Ca\(^{2+}\) Channel

PKC active

PKC inactive

Membrane Depolarization

Ca\(^{2+}\)

CCE

Sarcoplasmic Reticulum

ATPase

Ca\(^{2+}\) ATPase

Cytosolic

Ca\(^{2+}\)

ATPase

Fig. 15. Schematic diagram of a vascular smooth muscle cell showing several signaling pathways by which AI can elicit changes in cytosolic calcium concentration and contraction.

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CHAPTER III

EXPERIMENTS OVERVIEW

The experiments in this dissertation are divided into four sections. The overall hypothesis of the dissertation was the enhanced vasodilatory role of NO in the immature kidney is attributed to regulatory, expressional, and functional differences in the NOS isoforms from the adult. However, the overall hypothesis was broken into four parts to answer specific scientific questions with each of the four series of experiments in order to more thoroughly test the overall hypothesis.

The overall objectives of the dissertation was to: (1) determine which NOS isoform regulates immature renal hemodynamics by using functional whole animal studies utilizing intrarenal infusion of NOS inhibitors; (2) continue characterization of NOS expression in specific immature renal microstructures utilizing LCM (glomerular eNOS expression); (3) characterize developmentally regulated expressional and functional patterns of NOS isoforms, renin, and AT1 and AT2 receptors in the immature preglomerular resistance vasculature utilizing novel microdissection techniques; and (4) determine AII regulation of NOS expression and function in the immature renal vasculature using AT1 and AT2 receptor inhibitors.

All experiments involving animal usage, including euthanasia, fulfilled AVMA guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC).
FIRST SERIES OF EXPERIMENTS

The first series of experiments used *in vivo* whole animal models to study the effects of the NOS nonspecific inhibitor L-NAME and the nNOS specific inhibitor L-SMTC on the characteristics of postnatal renal hemodynamics, as compared to that of the adult. The effects of intrarenal perfusion of L-NAME on RVR, RBF and GFR were analyzed in both the neonate and adult kidneys. The L-NAME effects were compared to the effects of intrarenal perfusion of L-SMTC in both the neonate and adult to determine if the effects of nonspecific NOS inhibition were mediated by nNOS or eNOS. If intrarenal perfusion of L-SMTC causes alterations in RVR, RBF, and GFR that are comparable to that seen by L-NAME alone, than nNOS would be considered the primary NOS isoform responsible for the enhanced role of NO seen in the neonate. However, if the effects of L-SMTC were not comparable to that seen with perfusion of L-NAME, than eNOS would be considered the major NOS isoform responsible for the enhanced role of NO in the neonate.

Previous whole kidney studies by our lab have indicated L-NAME does cause an increase in RVR with subsequent decreases in RBF and GFR. However, L-SMTC studies have not been performed to further determine specific input by individual NOS isoforms in the neonate’s vasculature. Previous whole kidney expressional and protein studies by our lab have pointed to nNOS as the primary isoform leading to the enhanced role of NO in the immature kidney. However, *in vivo* physiological whole kidney functional studies using L-NAME and L-SMTC are required to determine if in fact nNOS is responsible for enhanced NO activity in the immature kidney. The experiments
in the first study of this dissertation were aimed at that specific goal. The objective of the first series of experiments was to intrarenally perfuse nonspecific NOS and nNOS specific inhibitors in whole animals to determine which NOS isoform is the major isoform regulating renal hemodynamics in the immature kidney. The hypothesis of the first series of experiments was nNOS is the major isoform regulating renal hemodynamics in the immature, but not the mature adult kidney.

SECOND SERIES OF EXPERIMENTS

Once the first series of experiments was completed, the next step was to determine exactly how NOS isoforms are regulated during development thus leading to the enhanced role of NO in the renal hemodynamics of the immature kidney. To determine this, localization and expressional abundance of NOS isoforms within specific vascular segments of the kidney were evaluated. Enhanced NO in the immature kidney may be a result of localization and expressional differences found in the postnatal kidney as compared to the adult. While whole kidney expressional and protein studies may suggest developmental regulation of NOS isoforms within the kidney, the exact location and abundance of NOS isoforms cannot be delineated as either vascular or tubular. To determine if developmental regulation of NOS isoforms occur in the kidney vasculature, where NOS could play a major role in renal hemodynamics, then specific vascular segments must be investigated. The second and third series of experiments in this dissertation were aimed at determining the expressional and functional contributions of the NOS isoforms in specific renal vascular segments.
Previous studies by our lab have observed developmental regulation of NOS expression in other kidney structures that have influence on renal hemodynamics, including the macula densa. However, no studies have been conducted to determine the developmental expression of NOS in the glomerulus. NOS would have an impact directly on GFR within the glomerulus through effects not only directed at vasoactive functions of the capillary beds within the glomerulus, but also through modulation of the ultrafiltration coefficient directly.

The second series of experiments were directed at determining the developmental regulation of eNOS in the glomeruli of the immature, as compared to the mature, kidney. In previous studies, it was determined nNOS is not present in the glomeruli and that eNOS is the major isoform found within the glomeruli. Furthermore, glomeruli developmental differences have been identified within different areas of the immature kidney. The juxtamedullary glomeruli develop before superficial glomeruli in the immature kidney, so differences in developmental regulation of NOS between different zones of the renal cortex were monitored in the second study as well. It is believed NO works to counterbalance All vasoconstriction and that All may modulate NOS expression through the AT1 receptor during development. To further investigate All modulation of eNOS within the glomerulus, the AT1 receptor inhibitor candesartan was intrarenally perfused and its effects on eNOS expression were analyzed. The second series of experiments utilized the dissecting technique LCM. The LCM technique is ideal for expressional studies involving dissection of glomeruli. However, LCM techniques were limited in the amount of sample that could be obtained so as a result, protein studies involving NO activity were omitted from glomeruli studies.
The objective of the second series of experiments was to compare endothelial nitric oxide synthase, eNOS, gene expression between superficial and juxtamedullary glomeruli during both basal conditions and angiotensin II type 1, AT1, receptor inhibition with candesartan using LCM to selectively harvest glomeruli from immature and mature porcine kidneys. The hypothesis of the second study was there are greater differences between superficial cortical and juxtamedullary glomerular eNOS expression in the immature kidney compared to the adult and that the AT1 receptor modulates expression of eNOS in the glomeruli of the newborn kidney, but not in the adult.

THIRD SERIES OF EXPERIMENTS

The third series of experiments were designed to investigate NOS isoform expression and function in preglomerular resistance vessels including the arcuate, interlobular, and afferent arterioles. The efferent arterioles account for 35% of the vascular resistance found in the kidney. However, the preglomerular resistance vessels are the location of 50% of the vascular resistance found within the kidney, and are the major site of regulation of RBF and GFR. In this third set of experiments, preglomerular resistance vessels were dissected from immature and mature kidneys and analyzed for NOS expression and function. Immature and mature kidneys were intrarenally perfused blue-dyed microspheres to stain specific preglomerular microvessels, which were then dissected from kidneys and analyzed for NOS isoform expression and NOS enzymatic catalytic activity. Again, since it is believed there is an
interaction between NO vasodilation and AII vasoconstriction, expression of RAS components in the preglomerular microvessels were also examined. Microdissection methods did not utilize LCM techniques in this third set of experiments for two reasons. The first reason was that LCM techniques are not conducive to obtaining kidney structures that are relatively large and of irregular shape, such as the resistance vessels. Secondly, the amount of sample obtained from LCM is relatively minute. In order to conduct NOS enzymatic catalytic assays, a fairly large amount of sample must be obtained.

The hypothesis of the third series of experiments was the enhanced role of NO functioning as a critical vasodilator is produced by developmentally regulated nNOS and eNOS expression and NO function in the immature renal vasculature, primarily in the resistance arterioles. These preglomerular resistance vessels were studied because they ultimately play the biggest role in determining RVR, RBF, and GFR. The objective of the study was to identify and compare the immature and mature expressional and functional differences of nNOS and eNOS in the renal vasculature, including the preglomerular resistance vessels arcuate, interlobular and afferent arterioles. Since it is believed there is an interactive counterbalancing between NO and the RAS, components of the RAS, including renin expression and AT1 and AT2 receptor expression, were also observed and compared.
The forth series of experiments were directed at determining the role of All as a regulator of nNOS and eNOS expression and function in the immature and mature renal resistance arterioles. Again, preglomerular microvessels, afferent arterioles, were dissected out of immature and mature kidneys with the visual aid of perfused blue-dyed microspheres. Once dissected, afferent arterioles were subject to experimental manipulation. Dissected vessels were administered All alone and in conjunction with candesartan, an AT1 receptor inhibitor, or PD 123319, an AT2 receptor inhibitor. Following experimental manipulation, microvessels were evaluated for NOS isoform expression and NOS enzymatic catalytic activity to see if All regulated these aspects of NOS through the AT1 and AT2 receptors.

The objective of the forth series of experiments was to determine the role of All as a regulator of nNOS and eNOS expression and function of NO production in the immature and mature renal resistance arterioles, primarily the afferent arteriole. The hypothesis of the study was that in the immature renal resistance vasculature, NOS expression and function are regulated by All through the AT1 and AT2 receptors.
CHAPTER IV

STUDY 1: NEURONAL NITRIC OXIDE SYNTHASE, nNOS, REGULATES RENAL HEMODYNAMICS IN THE POSTNATAL DEVELOPING PIGLET

INTRODUCTION

The immature renal vasculature is highly responsive to intrarenal NO stimulation with acetylcholine and NO inhibition with the non-selective NOS inhibitor L-NAME (126). Intrarenal infusion of L-NAME, in both whole animals, anesthetized piglets (131, 132), conscious lambs (121) and isolated perfused rabbit kidneys (124), produces significantly greater increases in RVR and decreases in RBF and GFR in the immature kidney than the adult. Since all previous studies in the developing kidney have utilized L-NAME, which non-selectively inhibits all NOS isoforms, it is not known which NOS isoform, either eNOS or nNOS, is regulating immature renal hemodynamics. Studies utilizing nNOS-specific inhibition with S-methyl-L-thiocitrulline (L-SMTC) suggest a renal hemodynamic role for nNOS in the adult rat during AII-induced hypertension (61) and diabetes (80). However, recent reports indicate that in the adult rat, nNOS may not play a role in basal renal hemodynamics. Kakoki et al., using two nNOS-specific inhibitors, L-NPA and v-NIO, recently reported
that nNOS does not regulate basal renal blood flow in adult rat (74). There are no studies utilizing nNOS specific inhibitors to examine developing renal hemodynamics.

Several characteristics of nNOS in postnatal renal maturation suggest that this isoform may importantly participate in renal hemodynamics after birth. Whole kidney nNOS gene and protein expression are greater in the developing piglet kidney than the adult pig throughout postnatal renal maturation, including at 21 days of age (128). nNOS expression also shows a distinct corticomedullary pattern in the developing porcine kidney. Cortical levels of nNOS protein during postnatal maturation, including the 14 to 21 day age range, are greater than the adult (128). Macula densa nNOS has a distinct postnatal maturational pattern in the postnatal maturing rat kidney (37). It is involved in the earliest stages of juxtaglomerular apparatus formation, and undergoes a pattern of extensive and abundant distal tubular localization, ultimately receding to the confines of macula densa in the adult. nNOS also demonstrates an interdependent morphologic relationship with the renin angiotensin system, RAS (37). The postnatal pattern of nNOS might have significance in relationship with the RAS. The RAS is highly activated at birth, with All a main vasoconstrictor (101, 140). There is an important renal hemodynamic regulatory interaction between NO and the RAS, perhaps mediated through the angiotensin II AT1 receptor sub-type (132). The AT1 receptor subtypes show a significant shift after birth, with the AT2 receptor, important for morphogenesis, decreasing after birth, with a concomitant increase in the AT1 receptor, which is involved in mediating vasoconstriction (10). Although there is emerging evidence that NO/All regulation of renal hemodynamics involves a balance of the activities of the vasoconstricting AT1 and the vasodilating AT2 receptors (20), this
receptor functional interplay has not been examined during postnatal maturation. In the immature kidney, the highly activated RAS with elevated AII (140) might mimic the intrarenal conditions seen in AII-induced hypertension in which nNOS is an important hemodynamic regulator (24, 61). Given the postnatal developmental characteristics of nNOS and the interplay with the RAS, we generated the hypothesis that nNOS is the major isoform regulating renal hemodynamics in the immature kidney, but not in the mature adult kidney.

Since there are no selective eNOS inhibitors available, participation of this isoform in these functional studies must be inferred by comparing the responses of non-selective inhibition with L-NAME to selective nNOS inhibition. In our previous studies, intrarenal infusion of L-NAME, the non-selective a competitive inhibitor of all NOS isoforms at 3μg/kg/min, a dose that does not alter mean arterial pressure, produces significantly greater increases in RVR and decreases in RBF and GFR in the 21 day old piglet with immature renal function than the adult pig (131, 132).

The objective of these studies, therefore, was to compare the renal hemodynamic responses to the intrarenal infusion of the selective nNOS inhibitor L-SMTC with the intrarenal infusion of the non-selective NOS inhibitor L-NAME in both the 21 day old piglets and adult pigs. The specific parameters used to evaluate renal hemodynamic responses to either L-NAME or L-SMTC included RVR, RBF, and GFR. In addition, urine flow and mean arterial pressure were observed to ensure conditions from experiment to experiment were consistent and systemic effects were absent.
MATERIALS AND METHODS

Subjects. Experiments were performed on mixed breed piglets with an average age of 21.5 days or adult pigs with mature renal function with an average age of 71.5 days. All groups received the same experimental preparation. As in our previous studies with developing piglets (131,132), all animals were fed a standard age-specific swine diet with normal sodium content (Southern States Cooperative, Inc., Richmond, VA). The 21 day old piglet was selected for study in these experiments. At this age, nephrogenesis is completed, and renal function is still immature (40, 100, 101, 131, 132). nNOS expression abundance is greater in the 21 day old piglet than the adult for both whole kidney protein and mRNA expression (128). Also, cortical nNOS is greater in the 14 day old piglet than the adult, an age window that includes the 21 day old piglet (128).

NOS inhibitors and age groups. These experiments utilized the selective nNOS inhibitor L-SMTC, which is easier to infuse into the renal artery due to its excellent water-soluble properties (62, 155). L-SMTC has been used in several studies examining the role of nNOS in renal hemodynamics including the blood-perfused juxtamedullary nephron preparation (62) or intrarenal infusion into the intact rat kidney (23, 61, 63). The dose of 0.027 μg/kg/min for 75 min in these experiments is based on studies using a L-SMTC intrarenal infusion (23, 142). The non-selective NOS inhibitor, L-NAME, at the dose of 3.0 μg/kg/min for 75 minutes was chosen based on our previous studies (131, 132). L-SMTC was infused in 21 day old piglets, n = 8, and adult pigs, n = 8. L-NAME was infused in 21 day old piglets, n = 8 and adult pigs, n = 8.
Preparation. All animals were fed a standard age-specific pig diet and were fasted overnight before experiments with access to water. All animals received initial anesthesia with intramuscular ketamine (adults 10 mg/kg and piglets 5 mg/kg) followed by i.v. sodium pentobarbital bolus, 10 mg/kg, and then sustaining anesthesia with intermittent i.v. sodium pentobarbital boluses, 0.5 mg/kg each. After tracheostomy, the animals were ventilated with small-animal respirator (Harvard Apparatus Co., Inc., S. Natick, MA), and constant low flow oxygen was given at 250-500 ml/min. Cannulas were placed in both external jugular veins for infusion of radioisotope and electrolyte solution. The left carotid artery was cannulated for monitoring MAP and blood sampling. The left kidney was exposed through a retroperitoneal flank incision and the left ureter was cannulated for timed urine collections. An electromagnetic flow probe (Carolina Instruments Electronics, Inc., King, NC) was placed around the renal artery to monitor renal blood flow. A 25-gauge right-angle needle was placed in the proximal renal artery for intrarenal infusions.

Experimental protocol. In the experimental design employed in these studies, each animal serves as its own control for the experimental infusion, obviating the need for separate time and vehicle controls. MAP and renal blood flow were monitored continuously throughout the experiment on a chart recorder (Grass Instrument Co., Quincy, MA). All animals received an i.v. maintenance infusion of 5% dextrose 0.45% sodium chloride (electrolyte solution) begun at 0.03 ml/kg/min. Glomerular filtration rate was determined by the clearance of 125 I-iothalamate (Glofil Isotex, Inc., Friends Wood, TX). A priming dose of 0.4 mBq/kg was followed by constant infusion throughout the experiment of 12 mBq at 0.3 ml/kg/h for adults and 2.4 mBq at 0.3
ml/kg/h for piglets. Intrarenal infusion of 0.9% saline solution at 0.1 ml/min was initiated. After 60 min recovery period, a 20 min control urine collection was obtained, along with a midpoint 3 ml blood sampling.

At the end of the control collection period, intrarenal infusion of L-NAME, L-SMTC, or vehicle, 0.9% saline (for time controls), began at the same rate, 0.1 ml/min. Both NOS antagonists were delivered in same solution as vehicle, 0.9% saline, L-NAME at a rate of 3.0 μg/kg/min, and L-SMTC at a rate of 0.027 μg/kg/min for 75 minutes. In all groups, the intrarenal infusion of L-NAME, L-SMTC, and vehicle were continued through a final 20 min experimental urine collection with a midpoint 3ml blood sampling. Blood and urine samples were evaluated for 125 I-iothalamate. The animals were euthanized with euthasol euthanasia solution, 2 ml i.v.

Statistics. As in our previous studies (131,132) experimental measurements were compared with control measurements using a paired t test. Differences between groups were determined by an unpaired t test. Statistical significance was considered to be p < 0.05. All data are expressed as mean ± SEM.

RESULTS

Neither L-NAME nor L-SMTC altered mean arterial pressure (MAP) from control in all age groups, as shown in Table 1. Control MAP in all 21 day-old piglet groups was significantly lower than the adult pig. L-NAME, L-SMTC, and control MAP values for piglets were significantly lower than adult values.
The effects on renal vascular resistance (RVR) of intrarenal infusion of the non-specific NOS inhibitor, L-NAME, or the nNOS selective inhibitor, L-SMTC, in piglets and adult pigs are shown in Fig. 16. As we have shown previously (32; 33), control RVR is significantly higher in both developing piglet control groups compared with the adult pig. The infusion of the non-selective NOS inhibitor, L-NAME, in the developing piglet produced RVR responses very similar to our previous studies (131, 132).

Following L-NAME infusion, RVR significantly increased from control level of 2.74 ± 0.07 mmHg/ml/min to 3.35 ± 0.11 mmHg/ml/min, a significant 22.7 ± 6.4% increase (Fig. 19). Similarly in the piglet, intrarenal infusion of the selective nNOS inhibitor, L-SMTC, increased RVR from 2.58 ± 0.27 to 3.76 ± 0.41, a 46.4 ± 8.6% increase (Fig. 19). The percent increase of RVR in response to infusion of L-NAME or L-SMTC was not statistically different. Intrarenal L-NAME infusion in adults also produced results similar to those we have previously reported (124, 125). Non-specific NOS inhibition with L-NAME significantly increased RVR from 1.24 ± 0.04 mmHg/ml/min to 1.39 ± 0.06 mmHg/ml/min, a 8.35 ± 2.6% change. In contrast, RVR in the adult pig did not

| Table 1. Effect of L-NAME and L-SMTC on MAP in piglets and adult pigs |
|-------------------|-------------------|-------------------|-------------------|
|                   | Control           | L-NAME            | Control           | L-SMTC            |
| Piglet            | 97.2 ± 2*         | 96.9 ± 3*         | 95.9 ± 5          | 96.3 ± 5*         |
| Adult             | 112.4 ± 3.8       | 115.3 ± 3.5       | 119.6 ± 2         | 120.9 ± 2         |

Values are means ± SE. N=8. *P < 0.05, = piglet control vs. adult. ‡P < 0.05 = piglet L-NAME and L-SMTC vs. adult.

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Fig. 16. The effects on renal vascular resistance, RVR, by intrarenal arterial infusion of L-NAME and L-SMTC in 21 day old piglets and adults. N=8. *P < 0.05, vs. control. ‡P < 0.05, vs. adult.

significantly change with L-SMTC intrarenal infusion, from $1.19 \pm 0.09$ mmHg/ml/min to $1.19 \pm 0.1$ mmHg/ml/min.

A similar pattern was seen in renal blood flow, RBF, responses to NOS inhibition with L-NAME or L-SMTC (Fig. 17). As previously demonstrated, the control period for both piglet groups RBF was significantly lower than the adult (131, 132). Intrarenal L-NAME significantly decreased RBF in the 21 day old piglet by $17.9 \pm 3.8\%$ (Fig. 19), from $1.57 \pm 0.07$ ml/min/gkw to $1.29 \pm 0.08$ ml/min/gkw. Similarly, the intrarenal infusion of L-SMTC significantly decreased RBF in the piglet by $30.4 \pm 3.8\%$ (Fig. 19), from $1.47 \pm 0.13$ ml/min/gkw to $1.02 \pm 0.10$ ml/min/gkw. Unlike the RVR responses, the RBF percent change in the piglet to L-SMTC was significantly greater than the L-NAME change. In the adult, L-NAME significantly decreased RBF from
Fig. 17. The effects on renal blood flow, RBF, by intrarenal arterial infusion of L-NAME and L-SMTC in piglets and adults. N=8. *P < 0.05, vs. control, ‡P < 0.05, vs. adult.

1.83 ± 0.11 ml/min/gkw to 1.73 ± 0.13 ml/min/gkw, a 7.57 ± 1.8% change (Fig. 19).

However, L-SMTC intrarenal infusion in the adult pig did not alter RBF.

Fig. 18 demonstrates the glomerular filtration rate, GFR, responses to intrarenal infusion of the NOS inhibitors. The control period GFR in both piglet groups was significantly lower compared to the adult, as seen in our previous reports (131, 132). L-NAME infusion in the piglet decreased GFR 30.3 ± 9.4% (Fig. 18) from 0.43 ± 0.04 ml/min/gkw to 0.30 ± 0.05 ml/min/gkw. Intrarenal L-SMTC produced a similar decrease in GFR in the piglet, 34.6 ± 10.5% (Fig. 18) from 0.40 ± 0.05 ml/min/gkw to 0.27 ± 0.06 ml/min/gkw. As we have previously reported, intrarenal L-NAME in the adult did not significantly alter GFR (124, 125). Additionally, GFR was not significantly altered in the adult pig after the intrarenal infusion of L-SMTC.
The percent change of RVR, RBF and GFR are compared between all the experimental groups in Fig. 19. The responses to L-NAME and L-SMTC in the 21 day old piglet were similar. Although the RVR increase of 46.4 ± 8.6% in the piglet was greater than the L-NAME increased RVR, 22.7 ± 6.4%, this was not statistically significant. However, the piglet RBF decrease in response to L-SMTC, 30.4 ± 3.8% was significantly greater than the L-NAME change of 17.9 ± 3.8%.

Neither L-NAME nor L-SMTC altered filtration fraction (FF) in either age group. For adults receiving L-NAME, FF remained unchanged from control, 0.30 ± 0.04 to L-NAME, 0.29 ± 0.03. Likewise, FF remained unchanged in adults receiving L-SMTC, from control, 0.30 ± 0.02 to L-SMTC, 0.32 ± 0.1. Similar results were seen in
Fig. 19. The percent changes of RVR, RBF and GFR by intrarenal arterial infusion of L-NAME and L-SMTC in piglets and adults. N=8. *P < 0.05, vs. adult, #P < 0.05, piglet LSMTC vs. L-NAME, ‡P < 0.05, adult L-NAME vs. L-SMTC.

piglets. FF in piglets receiving L-NAME did not change from control, 0.26 ± 0.02 to L-NAME, 0.24 ± 0.02, and in those receiving L-SMTC from control, 0.27 ± 0.2 to L-SMTC, 0.26 ± 0.02.

In time control experiments, both animal groups received intrarenal vehicle, 0.9% saline, in place of L-SMTC or L-NAME. Vehicle intrarenal infusion demonstrated no significant change in GFR, RBF, or RVR in either age group. Both L-NAME and L-SMTC caused significant reductions in urine flow volume in the piglet while only L-NAME decreased urine flow in the adult, as illustrated in Fig. 20.
DISCUSSION

This is the first description of a role for nNOS in the renal hemodynamics of the postnatal developing animal. Our previous studies demonstrated that intrarenal infusion of L-NAME produced significantly greater renal hemodynamic responses in the immature piglet kidney than the adult (131, 132). In these studies, intrarenal infusion of L-NAME in the adult, while not altering GFR, decreased RBF 9% and increased RVR 13%. The adult responses to L-NAME in these experiments were similar to the previous descriptions (Fig. 4). Using the same dose of, 3.0 μg/kg/min, the piglet responses to L-NAME in the current studies are similar to our prior reports, producing significant
reductions of GFR, 30.3%, and RBF, 17.9%, with an increase in RVR of 22.7%. There are no eNOS specific inhibitors, therefore we utilized the nNOS blocker, L-SMTC, to determine the respective contributions of the two isoforms. The intrarenal infusion of the nNOS inhibitor, L-SMTC, significantly changed renal hemodynamics in the piglet, compared to no changes in the adult. Further, the responses to nNOS inhibition were similar to those caused by non-specific NOS blockade with L-NAME, and as shown previously significantly greater than the adult pig responses to L-NAME. L-SMTC inhibition of nNOS in the immature kidney resulted in decreases in RBF, 30.4%, and GFR, 34.6% and increased RVR, 46.4%. Further evidence for the functional importance of nNOS in the immature kidney is provided by the urine flow rate (UV) responses. In the piglet, both L-NAME and L-SMTC produce similar, significant reductions in UV. However, in the adult, only L-NAME reduced UV, with no change in UV occurring with intrarenal L-SMTC. The intrarenal infusion of either NOS inhibitor did not produce systemic effects, such as alteration of blood pressure. In these experiments, as we have shown previously, base line renal function and MAP were significantly different between the piglet and the adult.

The responses to inhibition of the nNOS isoform seen in only the immature kidney in our experiments suggest a more important role for NO produced by nNOS during postnatal renal maturation. However, the role of nNOS in adult basal renal hemodynamics may not be as critical as in the developing kidney. NO plays a major role in the maintenance of RBF and GFR in a number of adult animal models in a variety of experimental conditions (83). Both systemic and intrarenal administration of competitive non-specific NOS inhibitors, such as L-NAME, leads to decreases in RBF.

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and, depending on the method, reduction in GFR (48, 87). However, Kakoki et al., using two nNOS-specific inhibitors, L-NPA and v-NIO, recently reported that nNOS does not regulate basal renal blood flow in adult rat, a finding similar to ours using L-SMTC in the adult pig (74). A major difference between the postnatal developing kidney and the adult in the basal state that might explain the responses to nNOS inhibition, is the highly activated RAS in the immature kidney, producing high intrarenal levels of All (140). The basal conditions of activated All in the developing kidney may be similar to pathophysiologic situations in the adult in which All is experimentally elevated. Under conditions in which All is elevated in the adult rat, All-induced hypertension (24, 61) and diabetes (80), nNOS-specific inhibition with L-SMTC produces significant renal hemodynamic changes. These experiments as were our previous studies (131, 132) were conducted in anesthetized animals, as compared to studies in conscious lambs (121). While anesthesia, per se, may activate vasoactive factors, such as All in the adult, the highly activated RAS in immature piglets is comparably elevated in both anesthetized (131, 132) and conscious (101) conditions relative to the adult. Thus in increased All conditions, either physiologic in the immature kidney, or pathophysiologic in the adult, nNOS appears to be an important hemodynamic regulator.

Further NO/All interaction is suggested by localization studies in the postnatal developing rat by Fischer et al. (37). The morphologic pattern of nNOS during nephrogenesis suggests an interdependent relationship with renin-producing cells in the juxtaglomerular apparatus. NO plays an important vasodilator role in counter-regulating the vasoconstriction of All (132). This functional interaction between NO and All may
be mediated by the AT1 receptor sub-type (132). The AT2 receptor, which is involved in nephrogenesis, undergoes a shift after birth such that the AT1 receptor mediating vasoconstriction becomes predominant (10). However, emerging evidence suggests that the AT2 receptor has a vasodilator function mediated by NO in the kidney with the effect of AT2 ultimately a balance between the two receptors (20). The functional interplay between AT1 and AT2 receptors and the interaction between AT2 receptors and NO in the postnatal renal kidney is not known.

This "neuronal" NOS isoform is readily available to participate in regulating renal hemodynamics (146). In the adult kidney, nNOS localizes predominantly to the macula densa, with additional expression in the afferent arteriole (92, 138). nNOS mRNA also is identified in microdissected renal vascular segments including the afferent arteriole (92). NO produced from nNOS participates in regulating the glomerular microcirculation (62) and glomerular filtration rate (122). The exact mechanism of macula densa nNOS regulation of afferent arteriolar tone is not known. Rather than directly diffusing into the afferent arteriole, NO from nNOS, acting within the macula densa, stimulates a cGMP-dependent signaling pathway producing vasodilation (111).

While there are no studies localizing or quantifying nNOS specifically in the renal resistance vasculature, several characteristics of nNOS in postnatal renal maturation suggest that this isoform may importantly participate in renal hemodynamics after birth. Whole kidney nNOS gene and protein expression are greater in the developing piglet kidney than the adult pig throughout postnatal renal maturation, including at 21 days of age (128). nNOS expression also shows a distinct cortico-
medullary pattern in the developing porcine kidney (128), where cortical nNOS is greater in the immature than mature kidney (128). The location of nNOS in the developing juxtaglomerular apparatus may contribute to vasoactive function. Although the direct localization of nNOS in resistance arterioles has not been performed, Fischer et al. demonstrated that macula densa nNOS undergoes maturational regulation in the rat kidney in a pattern that suggests an interdependent relationship with renin-producing cells (37). Thus, the developmental postnatal pattern of nNOS in both localization and abundance support the important role for this isoform in the immature kidney.

In summary, intrarenal arterial infusion of the nNOS-specific inhibitor, L-SMTC in the immature piglet resulted in significant changes in RBF, GFR and RVR that were similar to those seen with L-NAME. However, infusion of L-SMTC in the adult did not alter renal hemodynamics or function.

In conclusion, under basal, physiologic conditions, nNOS is an important regulator of renal hemodynamics and function in the immature kidney, but not in the adult, and may be the predominate vasoactive NOS isoform during postnatal renal maturation.
CHAPTER V

STUDY 2: THE DEVELOPMENTAL AND INTRACORTICAL PATTERN OF GLOMERULAR eNOS GENE EXPRESSION DURING PORCINE POSTNATAL RENAL MATURATION AND THE EFFECT OF AT1 RECEPTOR INHIBITION

INTRODUCTION

Fetal kidneys receive less than approximately 3% of cardiac output (103, 116). At the moment of birth, there is an immediate increase in renal blood flow characterized by a redistribution of blood flow from the inner cortex to the outer superficial cortex in newborns (2, 103). The distribution of intrarenal blood flow in the young, however, is different from that reported in the adult (108). The neonate kidney has a greater percentage of blood flow to the inner cortical and medullary areas than does the adult kidney (108). Blood flow to each region of the immature kidney increases with maturation (1, 19, 72, 79, 99, 18, 113, 114). The newborn receives approximately 10-15% of the cardiac output, however, this is still considerably less than the greater than 25% of the cardiac output the adult kidney receives.

In the weeks after birth, there is an associated rise in arterial pressure that contributes to an increase in renal blood flow with a corresponding decrease in renal vascular resistance (51, 72, 112). However, these factors do not appear to account for
the primary driving force behind the postnatal changes in renal hemodynamics because the rise in arterial pressure is of lesser magnitude than the rise in renal blood flow (108). Thus, it appears that other factors including structural/anatomical changes such as glomerulogenesis and vasculogenesis, physical factors such as autoregulatory response, and vasoactive factors such as the renin-angiotensin system contribute and possibly mediate the alteration in the neonate's renal hemodynamic state.

The contribution of structural changes to the maturational changes of renal vascular resistance in the developing kidney is an important consideration. Renal vascular resistance in the newborn kidney is a function of the number existing vascular channels, as well as the arteriolar resistance offered by each channel (30, 31, 70). The increase in renal blood flow in the immature kidney is influenced by structural changes in the kidney including the formation of new glomeruli, angiogenesis that accompanies nephrogenesis, and vascular remodeling. In swine, nephrogenesis continues after birth and is not complete until the third week after birth. However, renal blood flow continues to increase in species long after nephrogenesis and vasculogenesis is completed thus further implicating an enhanced role of vasoactive factors in the modulation of the neonate's renal hemodynamic state (60, 72, 113, 116). The neonate's renal vasculature does undergo hypertrophic outward remodeling and contributes to a rise in renal blood flow. The increase in the diameter of resistance vessels during maturation is greater in the kidney than in any other organ, however, renal vascular resistance remains elevated to a greater extent than in any other organ. Taken collectively, morphological changes in renal resistance vessels cannot account for the change in renal blood flow that occurs in the period of renal hemodynamic maturation.
Recent studies indicate the functional vasoactive characteristics of the immature renal vessels determine the decrease in renal vascular resistance that, in turn, dictates renal blood flow. Although structural and physical development may contribute to the changes in the developing kidney by reducing renal vascular resistance, the functional vasoactive factorial changes that occur within the renal microvasculature are the main factors that produce the maturational changes in renal hemodynamics.

In the developing porcine kidney, glomerular maturation increases from immature superficial to more advanced juxtamedullary nephrons. Numerous studies have found that the major NOS isoform within glomeruli contributing to the neonate’s hemodynamic state is the endothelial NOS isoform (eNOS). Our lab has previously shown that eNOS undergoes distinct whole kidney and corticomedullary developmental regulation during postnatal maturation (129). However, the postnatal developmental pattern of specific renal structures, such as the glomerulus, is unknown. In the postnatal developing porcine kidney, which maintains nephrogenesis until 21 days of age, individual glomerular maturation increases from immature superficial cortical to more advanced juxtamedullary nephrons. The hypothesis of this study is that there are greater differences between superficial cortical and juxtamedullary glomerular eNOS expression in the immature kidney, compared to the adult, and that the AT1 receptor modulates expression of eNOS in the glomeruli of the newborn kidney, but not in the adult. The objective of this study was to compare endothelial nitric oxide synthase, eNOS, gene expression between superficial, SG, and juxtamedullary, JMG, glomeruli during both basal conditions and angiotensin II type 1, AT1, receptor inhibition with
candesartan using Laser Capture Microdissection, LCM, to selectively harvest glomeruli from immature and mature porcine kidneys.

The experimental method used in this glomerular studied utilized LCM, which permits direct microscopic visualization and one-step procurement of selected cell populations from a slide-mounted section of complex tissue, such as the kidney. The method involves placing a thin, transparent transfer film over a tissue section on a standard histopathology slide, visualizing the tissue microscopically, and selectively adhering the cells of interest to the transfer film with a fixed-position, short-duration, focused pulse from an infrared laser. The brief, mild thermal transients experienced by the tissue in the capture process do not damage DNA, RNA, or proteins. The transferred tissue, firmly embedded in the transfer film, is accessible to aqueous solutions necessary for further analysis.

MATERIALS AND METHODS

Animals. All animals were fed a standard age-specific pig diet and were fasted overnight before experiments with access to water. The animals used were mixed breed piglets and adults (Smithfield, VA). Piglets were ages representative of the postnatal developmental spectrum, newborn, 7, 14 and 21 days. Three to four animals per age group were used for each experiment. Experiments were repeated on animals in order to obtain statistical significance.
Basal glomerular NOS expression. Animals were sacrificed with a lethal dose of euthasol (1.5 ml / kg or 10 mg / kg) (Virbac, Fort Worth, Texas) and kidneys were exposed and excised through a retroperitoneal flank incision.

Glomerular NOS expression after AT1 receptor inhibition. All animals received initial anesthesia with ketamine intramuscularly (adults 10 mg / kg; piglets 5 mg/kg) followed by intravenous sodium pentobarbital bolus (10mg / kg). An anesthesia was sustained with intermittent intravenous sodium pentobarbital boluses (0.5 mg / kg each). Following tracheotomy, animals were ventilated with an animal respirator (Harvard Apparatus Co., Inc., U.S.A.) with constant low flow oxygen administered at 250-500 ml/min. Cannulae were placed in both external jugular veins for infusion of maintenance solution (5% dextrose, 0.33% normal saline) at a rate of 0.03 ml/kg/min. The left carotid artery was cannulated and used to monitor mean arterial pressure (MAP) and blood sampling. The left kidney was exposed through a retroperitoneal flank incision. A 25-gauge right angle needle was placed proximal in the renal artery for intrarenal infusions. Kidneys were initially perfused with 0.9% saline solution at 0.1 ml/min for 20 min followed by perfusion of the AT1 receptor antagonist candesartan at a rate of 1 ug/kg/min for 60 minutes. Candesartan administered at 1 ug/kg/min has been shown not to change systemic blood pressure. A series of preliminary experiments (n=3) were conducted to obtain the optimal infusion time of candesartan.

Sampling. After removal, kidneys were decapsulated, snap frozen in O.C.T. fixing compound (VWR, Bridgeport, NJ), and stored at -80°C. Cortico-medullary 10 um slices were cut from the frozen kidneys and stained with the Arcturus HistoGene Staining Kit (Arcturus, Mountain View, Calif.) immediately prior to Laser Capture Microdissection,
LCM, sampling. The system used for LCM was the PixCell Ile Laser Capture Microdissection System (Arcturus, Mountain View, CA). During LCM microdissection, glomeruli were chosen for dissection based on location within the cortex, either superficial or juxtamedullary.

**RNA isolation and RT-PCR.** Each sample consisted of 80 dissected glomeruli. The desired glomeruli were selectively adhered onto transfer caps (Arcturus, Mountain View, CA) during LCM microdissection, then placed in a clean 0.5 ml microtube containing GITC lysis buffer (Qiagen, Inc., Valencia, CA) with 10 μl/ml μ-mercaptoethanol and subject to immediate isolation of RNA using QIAshredders and the RNeasy kit (Qiagen, Inc., Valencia, CA). Isolated RNA from LCM-obtained glomeruli was concentrated via vacuum centrifugation. Concentrated RNA was used to synthesize cDNA. For removal of mRNA secondary structures, 5 μg of isolated RNA was incubated for 3 minutes at 70°C with 5X reaction buffer (Invitrogen, Carlsbad, CA), which contained 250 mM Tris-HCl (pH 8.3), 375 KCl, and 15 mM MgCl₂. After removal of secondary structures, RNA was hybridized with 2 μl of random primers (Applied Biosystems, Alameda, Calif.), 2 μl of DTT (Invitrogen, Carlsbad, CA), 1 μl of 10 mM dNTP (containing 10 mM each of dATP, dGTP, dCTP, and dTTP) (Invitrogen, Carlsbad, CA), 0.5 μl of RNasin (Promega, Madison, WI), and 0.5 μl of MI-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) in a 20 μl reverse transcriptase reaction mixture. The reaction was carried out in a thermocycler (Biometra, Bigetten, Germany) at 25°C for 5 min, 37°C for 1 hr, and 70°C for 10 min. The cDNA was analyzed for eNOS mRNA gene expression by real-time RT-PCR (Lightcycler, Roche Applied Science, Indianapolis, IN) using the LightCycler FastStart DNA Master SYBR Green I
amplification kit (Roche Applied Science, Indianapolis, IN). Individual reactions were 20 ul in total volume which included: 50 ng of RT reaction, 10 uM of each primer, 2.5 mM MgCl₂, and 10 ul of LightCycler FastStart DNA Master SYBR Green I amplification mix. Primers used were specific for eNOS and 18s rRNA. Following an initial 15 minute preheating period at 95°C for polymerase enzyme activation, polymerase amplification was carried out for 40-45 cycles (94°C denaturing cycle for 15 sec., 55°C annealing cycle for 30 sec., and 72°C extension cycle for 10-20 sec.). The PCR products were separated and visualized by electrophoresis in agarose gel containing ethidium bromide under UV light to confirm product specificity. Porcine specific primers were used to amplify a 190 bp region within the eNOS gene (1495-1684 bp). Primer sequences for eNOS were (sense) 5'-GAGCACAGAGATTGGC-3' and (antisense) 5'-GTCCACAATGGTCACTTT-3'. 18s rRNA was used as an internal reference. The expression of 18s rRNA is an effective internal reference because it does not undergo significant regulation in the postnatal immature kidney. The 18s rRNA primer sequences were (sense) 5'-GGGCATTCGTATTGCG-3' and (antisense) 5'-AGCTATCAATCTGTCAATCCT-3'. The 18s rRNA primers amplified a 371 bp region within the 18s rRNA gene (176-546 bp).

Standard curves. To generate eNOS and 18s rRNA cDNA standard curves, eNOS and 18s rRNA PCR products were separated by agarose gel electrophoresis. The products were purified from the gel using a gel extraction kit (Qiagen, Inc., Valencia, CA), quantitated by measuring absorbance at 260 nm, and used as DNA templates for quantifications using real-time RT-PCR. Products of varying concentrations were subject to amplification and subsequent real-time RT-PCR crossing points were used.
for generation of standard curves. Product concentrations were placed on the y-axis while crossing points were placed on the x-axis for standard curves of all cDNA genes studied.

Statistical analysis. Experiments were repeated x 7. All data are reported as means ± SE. Differences between age groups were determined by an unpaired t test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Superficial Glomeruli

Basal expression of eNOS mRNA was observed in superficial glomeruli of porcine kidneys of all ages (Fig. 21). The lowest eNOS expression was observed in newborn and 7 day old kidneys. The expression of eNOS rose sharply at 14 days where

![Graph showing expression of eNOS mRNA in superficial glomeruli in porcine ages newborn, 7, 14 and 21 days, and adult. Results normalized to 18s rRNA. N=7. *$p < 0.05$, vs. newborn. +$p < 0.05$, vs. 7 day old.](image)
there was observed an approximately eight fold increase in expression. Expression of eNOS continued to increase in superficial glomeruli with maturation. Greatest eNOS expression was observed in the 21 day old and adult kidney. Adult and 21 day old eNOS expression was ten times greater than newborn and 7 day old expression.

Juxtamedullary Glomeruli

The basal expression of eNOS mRNA was also observed in juxtamedullary glomeruli of porcine kidneys of all ages (Fig. 22). Juxtamedullary glomerular eNOS was significantly greater than superficial glomerular eNOS only in the newborn immature kidney. The expression of juxtamedullary glomerular eNOS dropped six fold after birth to reach its lowest levels at 7 days old. Proceeding the decline in expression seen in the 7 day kidney was a twelve fold increase which was observed in the 14 day kidney. The level of eNOS expression maintained consistent values from 14 day through adulthood with only a slight decline in expression at 21 days. Adult kidneys

![Graph](image-url)

Fig. 22. Expression of eNOS mRNA in juxtamedullary glomeruli in porcine ages newborn, 7, 14, and 21 day-old, and adult. Results normalized to 18s rRNA. N=7. +P < 0.05, vs. 7 day old.
exhibited the greatest levels of eNOS expression in juxtamedullary glomeruli. Adult eNOS expression was almost twice that seen in the newborn kidney and fifteen times greater than that observed in the 7 day kidney.

**Total Glomeruli**

Total glomerular eNOS mRNA expression followed our previously described eNOS whole kidney expressional pattern that was characterized with a dip at 7 days, and greater expression observed in the 14 day, 21 day, and adult kidneys (Fig. 23). Newborn eNOS expression was comparable to eNOS expression in the more mature animals. At 7 days, eNOS expression decreased by almost four fold. After 7 days, eNOS expression increased six fold as seen in the 14 day old kidney. After 14 days, eNOS expression remained constant with maturation with a slight increase in the adult kidney. Greatest expression was observed in the adult kidney.

![Fig. 23. Expression of eNOS mRNA in total glomeruli (superficial + juxtamedullary) in porcine ages newborn, 7, 14 and 21 day old, and adult. Results normalized to 18s rRNA. †P < 0.05. vs. 7 day old.](image)

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AT1 Receptor Inhibition in Superficial Glomeruli

The renal perfusion of the AT1 receptor inhibitor candesartan resulted in significant blunting of eNOS mRNA expression in the superficial glomeruli of newborn, but not adult (Fig. 24). In the newborn, administration of candesartan resulted in an almost twenty-five fold decrease in eNOS expression. The expression of eNOS in the adult was very low in both control and experiment kidneys, as compared to the newborn control. Adult levels of control eNOS were significantly lower than that in the newborn control. There was no statistical difference between control and experiment values in the adult kidneys when candesartan was administered.

AT1 Receptor Inhibition in Juxtamedullary Glomeruli

Renal perfusion of candesartan significantly attenuated eNOS expression in juxtamedullary glomeruli of the newborn but not in the adult (Fig. 25). When given...
candesartan, the newborn experienced a six-fold decrease in eNOS expression. Adult control levels of eNOS expression were significantly lower than newborn control levels. In the adult kidney, candesartan did not statistically effect eNOS expression.

![Graph showing expression of eNOS mRNA](image)

**Fig. 25.** Expression of eNOS mRNA juxtamedullary glomerulus in porcine ages newborn, 7, 14 and 21 days, and adult in control animals and animals renally perfused the AT1 receptor inhibitor candesartan. Results normalized to 18s rRNA. N=7. +*P < 0.05, vs. newborn AT1X, adult control, and adult AT1X.

**DISCUSSION**

The distribution of intrarenal blood flow in the young is different from that reported in the adult (108). The neonate kidney has a greater percentage of blood flow to the inner cortical and medullary areas than does the adult kidney (108). The inner cortex is the site of juxtamedullary glomeruli while the outer cortex contains the superficial glomeruli. In the superficial areas of the cortex, nephrogenesis and
angiogenesis are still occurring in the newborn porcine kidney. Thus, glomeruli and the accompanying vasculature are not fully operational in the superficial areas of the cortex. As a result, blood flow is routed to areas of the kidney that contain glomeruli that are fully functional. The glomeruli within the juxtamedullary zone are functional and so the majority of blood flow is directed to these glomeruli. This would explain why a greater percentage of blood flow goes to the inner cortical and medullary areas in the newborn as compared to the adult. The intrinsic mechanisms that occur during development to modulate intrarenal blood remain unstudied.

In the results of this study, newborn juxtamedullary glomeruli exhibited a high level of eNOS expression when compared to newborn superficial glomeruli. This would suggest NO may be required for the increase in renal blood flow that is directed toward the inner cortex. The glomeruli are the sites of filtration within the kidney, so blood flow through the glomerulus is critical. While the main sites determining renal blood flow are the preglomerular microvessels, the capillary beds within the glomeruli can also effect renal blood flow and directly influence filtration within the Bowman’s capsule. While NO effects the vasoproperties of the glomerulus’ capillary bed, NO can have direct effects on glomerular filtration properties, including alterations in the ultrafiltration coefficient.

The increase in renal blood flow to the neonate’s inner cortical region and juxtamedullary glomeruli would suggest an increase in the need for vasodilators such as NO in that specific cortical area. This was observed in the newborn’s juxtamedullary glomeruli data provided in this study. eNOS expression was relatively high in the newborn’s juxtamedullary glomeruli. On the other, with low blood flow, the newborn’s
outer cortex would not require vasodilators. This was also seen in this study where newborn superficial glomeruli demonstrated low levels of eNOS as compared to juxtamedullary glomeruli.

As the kidney continues to develop and nephrogenesis nears completion, blood flow distribution widens to increase flow to the outer cortex to newly functioning superficial glomeruli. In the data presented in this study, after 14 days, eNOS expression is fairly constant throughout the rest of maturation in both juxtamedullary and superficial glomeruli. The 14 day age may mark the period when most renal glomeruli, both juxtamedullary and superficial, become fully functionable and capable of handling plasma filtration loads comparable to that seen in the adult. At 14 days, vasoactive substances, particularly vasodilators, in the vasculature in all renal cortical zones, both inner and outer, are required to reach normal physiological renal function.

Interestingly, there is a significant decrease in eNOS expression in both juxtamedullary and superficial glomeruli at 7 days. In whole kidney eNOS studies involving porcine, the same expressional dip was observed in 7 day old animals. The significance of the drop in eNOS expression at 7 days remains unexplained. One theory is that there is a transition that occurs at 7 days post birth. Maternal programming may ready the fetus’s renal hemodynamic state for birth, then during and immediately after birth, the renal hemodynamics of the newborn may be directed through mechanisms that acclimate the newborn’s kidney to function without maternal input. Once the immediately newborn period passes, the newborn may undergo another renal transition at 7 days post-birth in which the developing kidney undergoes intrinsic transitional mechanisms in order to take on adult like renal hemodynamics to begin functioning.
with adult renal capabilities. A factor that may be a signaling mechanism, or just a part of the overall process, may include a reduction in eNOS expression as seen in the 7 day old in both juxtamedullary and superficial glomeruli. Whatever the reason, the decrease in eNOS expression seen in the 7 day old kidney, both on the whole kidney level and on the level of glomeruli, requires further investigation.

As discussed previously, the increased RVR found in the developing kidney is a result of the enhanced vasoconstrictor response of AII. In this study, blockade of the AT1 receptor inhibited eNOS expression in both juxtamedullary and superficial glomeruli of the newborn, but not of the adult. This suggests eNOS in both the juxtamedullary and superficial glomerulus are regulated by AII through the AT1 receptor. In the inner cortical area of the developing kidney, NO may be required to overcome the AII induced increase in RVR to allow renal blood flow to functioning juxtamedullary glomeruli for filtration. Even though blood flow to superficial glomeruli is low immediately after birth, AII serves to regulate eNOS expression through the AT1 receptor. However, the decrease in renal blood flow to the outer cortex may not be a result of enhanced AII vasoconstriction because basal eNOS levels are low in superficial glomeruli even though it was observed in this study that AII regulates eNOS expression in superficial glomeruli through the AT1 receptor. Thus, other mechanisms, mainly structural, may account for the low blood flow to outer cortical areas of the newborn kidney. However, once those structural factors are overcome, AII regulates eNOS expression in the superficial glomeruli of the developing kidney. Once the kidney has matured, it appears from the adult data provided in this study, AII does not regulate eNOS expression in neither the adult juxtamedullary nor superficial glomeruli.
The regulation of eNOS expression and translation appears to be complex in the neonate. This study provides evidence for AII regulation of eNOS expression in the glomeruli of the neonate. Other factors appear to contribute to the regulation of eNOS in the newborn as well. Estrogen has been found to upregulate eNOS mRNA and protein expression (129). Immediately after birth, the newborn has high levels of estrogen and estrogen receptor (129). Estrogen may influence glomerular eNOS expression. Two growth factors present in the neonate have also been found to regulate eNOS expression. Transforming growth factor-β1, which is highly expressed during renal development, upregulates eNOS (129). Vascular endothelial growth factor (VEGF)-A also upregulates eNOS. VEGF-A is a participant in nephrogenesis and angiogenesis during the formation of glomerular vessels (129). In regard to neonate renal hemodynamics, the role of eNOS may be critical in modulating the vasoconstrictor response of AII and thus is regulated by the AT1 receptor in the renal vasculature. However, there are other potential factors relevant to the regulation of eNOS in the developing kidney including cell growth and proliferation, hormones such as estrogen, and growth factors.

It is important to remark on one of the differences found between basal eNOS and AT1X control eNOS expressional values. In the basal experiments, eNOS expression in both juxtamedullary and superficial glomeruli in the newborn was lower than that found in the adult. However, in the AT1X experiments, control levels of juxtamedullary and superficial glomeruli eNOS were higher in the newborn than in the adult. This may be explained by differences in experimental methods. In the basal experiments, animals were sacrificed and kidneys were immediately removed,
glomeruli were dissected, and eNOS expression was analyzed promptly. The procedure for AT1 inhibition required a longer experimental period. Animals were anestitized and candesartan was intrarenally perfused for 60 minutes. Effects from anesthesia and prolonged experimental period required for candesartan perfusion may account for the discrepancies seen between basal and AT1X control eNOS expression.

While other studies have described the absence of nNOS in the glomeruli and the presence of eNOS in glomeruli in adult kidneys, this is the first study that has observed glomerular eNOS expression in the developing kidney. The data in this study demonstrated eNOS glomerular expression is developmentally regulated with differences observed between juxtamedullary and superficial glomeruli and between neonate and adult glomeruli. In the newborn kidney, juxtamedullary glomeruli exprience high eNOS expression compared to low superficial glomerular eNOS expression. There is a significant decrease in eNOS expression in both juxtamedullary and superficial glomeruli in the 7 day old kidney. At 14 days, eNOS expression increases and maintains a fairly constant level of expression throughout the rest of maturation to adulthood in both juxtamedullary and superficial glomeruli. Blockade of the AT1 receptor inhibited eNOS expression in both juxtamedullary and superficial glomeruli of the newborn, but not in the adult. The data from this study confirms the hypothesis that there are greater differences between superficial cortical and juxtamedullary glomerular eNOS expression in the immature kidney, as compared to the adult, and that the AT1 receptor modulates expression of eNOS in the glomeruli of the newborn kidney, but not in the adult.
CHAPTER VI

STUDY 3: NITRIC OXIDE SYNTHASE, RENIN AND ANGIOTENSIN II RECEPTOR EXPRESSION AND NITRIC OXIDE PRODUCTION IN THE RENAL RESISTANCE VASCULATURE DURING PORCINE POSTNATAL DEVELOPMENT

INTRODUCTION

The uniqueness of the neonate's renal hemodynamics impacts newborn clinical management, and predisposes the immature kidney to develop vasomotor-mediated acute renal failure due to a variety of perinatal pathologies such as hypoxia, ischemia, shock, and prematurity complications. Newborn RBF is low relative to the adult. Thereafter, RBF progressively increases during postnatal maturation to adult levels. At the moment of birth, there is an immediate increase in renal blood flow characterized by a redistribution of blood flow from the inner cortex to outer superficial cortex (2, 103). The predominant factor contributing to the low RBF is high RVR. The increase in RBF during maturation is due to reductions in RVR. There is an associated rise in systemic arterial pressure that contributes to the increase in renal blood flow while renal vascular resistance continues to decrease, but the rise in arterial pressure is of lesser magnitude than the rise in renal blood flow (51, 72, 112). Thus, it appears that other factors
including structural / anatomical changes, physical factors, and vasoactive factors mediate the alteration in the neonate’s renal hemodynamic state.

RVR in the newborn kidney is a function of the number of existing vascular channels, as well as the arteriolar resistance offered by each channel (30, 31, 70). RBF continues to increase in species long after nephrogenesis and vasculogenesis is completed thus further implicating an enhanced role of vasoactive factors in the modulation of the neonate’s renal hemodynamic state (60, 115, 131). Taken collectively, morphological changes in renal resistance vessels cannot account for the change in RBF that occurs in the period of renal hemodynamic maturation.

Many studies have indicated the primary vasoactive factor that influences neonate RVR is the RAS. All components of the RAS are highly expressed in the developing kidney. An intact and functional RAS are required for normal kidney development. Pharmacological blockade of the RAS in developing kidneys has been found to cause papillary atrophy, abnormal wall thickening of intrarenal arterioles, tubular atrophy associated with expansion of the interstitium, impairment in urinary concentrating ability, and impairment of nephrogenesis (7). All has both hypertrophic and proliferative effects on the various cell types found within the kidney (60). Under most circumstances, renin secretion is the rate-limiting step in the production of All (108).

During development, renin and subsequent All levels are very high. In fact, fetal angiotensin levels are twice that as found in the maternal plasma (108). Circulating levels of All remain high during the newborn period and decrease with age paralleling the changes in active renin (108). The enzymes that are responsible for All degradation
are relatively inactive in the developing animal, and therefore, high levels of circulating All are maintained during early life (108). The major components of the RAS are highly present in the immature kidney but their anatomical distribution and activities are quite different than those seen in the adult kidney. In immature rats, renin is found to be expressed throughout the renal preglomerular vasculature including the arcuate, interlobular and afferent arterioles (44). Upon maturation, renin expression is confined to structures more localized around the juxtaglomerular apparatus.

All effects are mediated through two receptors in the kidney, the AT1 and AT2 receptors. In general, studies have found the AT1 receptor to be of low abundance at birth and increases in expression with maturation while the AT2 receptor declines with postnatal age. The functions of the AT1 and AT2 receptors during fetal and neonate development remain incompletely studied.

The potent vasoconstrictor response of All remains intact in the newborn and appears to be the major factor contributing to the high RVR found in the immature kidney. The 86% decrease in RVR that occurs during maturation is attributed to a reduction in the distribution and abundance of All and its receptors in the preglomerular vasculature (51). The intrarenal infusion of a non-peptide AT1 receptor antagonist produces greater increases in RBF in newborn piglets than in adults, demonstrating that All is a more important vasoconstrictor in the developing kidney than in the adult (132). Unchecked, the potent vasoconstrictor response of highly active All in the newborn can lead to adverse renal complications including ARF.

As discussed in earlier chapters, NO is an important regulator of renal hemodynamics in the immature kidney, and functions as a critical vasodilator to
counterbalance highly activated AII (132). The immature renal vasculature is highly responsive to alterations of NOS. The intrarenal infusion of the NOS inhibitor L-NAME in piglets and adult swine produces greater renal hemodynamic responses in the newborn than in the adult (121, 131). This data suggest that renal NO production by NOS is greater in young animals than in adults. This increase in NO production is supported by the distinct developmental patterns in the postnatal maturing porcine kidney (128, 129). Furthermore, studies by Han et al. showed that eNOS expression in rats is elevated at birth, decreases temporarily, then rises up to adult levels with continued maturation (53). Studies by Mattson et al. identified both nNOS and eNOS isoforms in the preglomerular vessels of the adult rat kidney including the arcuate, interlobular, and afferent arterioles, a finding that supports similar observations from other laboratories (7, 141). However, no studies have investigated the presence of either NOS isoform in the neonate’s vasculature. NO is believed to counterbalance the highly activated RAS and protect the immature kidney from the deleterious effects of adverse perinatal events that lead to vasomotor ARF (108).

The exact relationship between AII and NO and the ability of NO to buffer AII vasoconstriction has not been elucidated in the renal vasculature of the immature kidney, particularly in the preglomerular resistance vasculature including the afferent, interlobular and arcuate arterioles. In the adult rat, the vasoconstrictor response to AII in the preglomerular resistance vasculature was enhanced in eNOS knock out mice (105). AII upregulated NO production via the AT1 receptor in the preglomerular resistance vasculature of rats, specifically in the afferent arteriole (104). AT1 receptor inhibition inhibited AII stimulated NO production in the renal arteries of adult rats (135). Recent
studies clearly indicate there is an immediate relationship, or interaction, between NOS isofom expression, NOS enzymatic catalytic activity (thus leading to NO production), and AII in the adult kidney. Numerous studies have indicated renal AII levels are high in the fetus and neonate, and is required for normal renal development. With increased renal AII levels, vasoconstriction within the preglomerular resistance vasculature is also enhanced. Upregulation of NOS expression and function maybe the mechanism by which the preglomerular resistance vasculature of the immature kidney counterbalances increased vasoconstrictor activity. However, AII and NO interaction has yet to be studied in the renal vasculature of the immature kidney.

The hypothesis of this study was the enhanced role of NO as a critical vasodilator, counteracting highly activated constrictors such as AII during postnatal renal maturation, is produced by developmentally regulated nNOS and eNOS expression and NO function in the immature renal vasculature, primarily in the resistance arterioles. These preglomerular resistance vessels were studied because they ultimately play the biggest role in determining RVR, RBF, and GFR. The objective of the study was to identify and compare the immature and mature expressional and functional differences of nNOS and eNOS in the renal vasculature, including the preglomerular resistance vessels arcuate, interlobular and afferent arterioles. Since it is believed there is an interactive counterbalancing between NO and the RAS, components of the RAS, including renin expression and AT1 and AT2 receptor expression, were also observed and compared.
MATERIALS AND METHODS

Microdissection of Microvessels

Animals. These experiments utilized the preparation and study of kidney slices from ages representative of the developmental spectrum, newborn, 7, 21 day piglets and adults. All experiments were performed on mixed breed swine obtained from the same local vendor (Smithfield, VA). All animals were transported and housed under the same conditions. Kidneys of pre-weanling piglets (newborn and 7 days old) were harvested on arrival. Kidneys of weaned piglets (21 days old) and adults >70 days of age were harvested 24 hr after arrival to allow for dietary and climatic adjustment. Animals were fed a standard age-specific pig diet and were fasted overnight before experiments with access to water.

Preparation. Animals were initially anesthetized with intramuscular ketamine (adults 10 mg/kg; piglets 5 mg/kg), followed by a sacrificial intravenous euthasol infusion (50 mg/kg each) (Delmorva Laboratories, Midlothian, VA). The left kidney was exposed through a retroperitoneal flank incision. The aorta was tied closed both proximal and distal from the renal artery branching from the aorta. A 20 - 25 gauge right angle needle was placed in the proximal renal artery for intrarenal perfusion.

Microdissection of renal microvessels. The techniques were modified from previous publication (106). The left kidney was perfused with 10 ml of 37°C dissecting solution (DS) at a rate of 1 ml per minute (10 minute total DS perfusion period). The DS consisted of (in mM) 135-140 NaCl, 3-5 KCl, 1.5 CaCl₂, 1-1.2 MgSO₄, 5-5.5 glucose, 5 HEPES (pH 7.4), 10 sodium acetate, 5 L-alanine, and 5 L-succinic acid (Sigma, St.
Louis, MO). Following perfusion of DS, kidneys were perfused with 2 – 3 ml of polybead polystyrene blue dyed microsphere mixture containing particles of 0.2, 0.5, 1.0, 3.0, 6.0 and 10 um in diameter (Polysciences, Warrington, PA). Polybeads were incubated to 37°C prior to infusion. Microspheres were in solution with 10% bovine serum albumin (Sigma, St. Louis, MO). After decapsulation and removal of the collecting system, the kidney was excised and cut into 500-600 um coronal slices that contained the entire cortico-medullary axis (McIlwain tissue chopper, The Mickle Laboratory Engineering Company). Coronal slices were placed into DS containing 0.7 – 2 mg/ml collagenase (192 U/mg; Worthington Biochemical, Freehold, NJ), 1 mg/ml dithiothreitol (Invitrogen, Carlsbad, CA), and 1 mg / ml BSA and incubated on a shaker at 37°C with 95 % oxygen and 5 % carbon dioxide for 25-30 minutes. Kidney slices were rinsed twice with collagenase free DS to remove any collagenase. Kidney slices were then placed in a petri-dish containing ice-cold DS, 5 – 10 mM vanadyl ribonucleoside complex (Sigma, St. Louis MO), 0.1 mg/ml trypsin inhibitor (Sigma, St. Louis MO), 5 mM dithiothreitol, and 1 % BSA. The petri-dish was placed on a stereomicroscope stage (Bausch and Lomb, Rochester, NY) and the slices/solution were maintained at 4°C during microdissection. Microscopy settings for microdissection included using dark field illumination and magnification ranging from 16 – 210x. Dissection of various intrarenal vessels included using #5 dissecting forceps, iris scissors, glass pipettes rinsed in 10% BSA, suture forceps, and 30 – 33 gauge stainless steel needles. Dissecting time was restricted to a maximum of 1.5 hrs. During dissection, 10–20 mm (measured via the microscopes eye piece micrometer) of microvessels were obtained. Dissected microvessels were placed on the side of the
petri-dish for removal of unwanted segments. Microvessels were rinsed with fresh dissecting solution containing 2 U/ul RNasin (Promega, Madison WI) and 5 mM DTT. Dissected vessels were transferred to a small microcentrifuge tube containing 10 ul of ice-cold DS and centrifuged at 12,000 g for 2 min. Supernatant was discarded. Lysis buffer was added to dissected vessels and placed on ice (4°C) for 60 minutes to permeabilize the microvascular cells. RNA isolation was started following the 60-minute lysis period.

Tissue Analysis Techniques for mRNA studies

Once microvessels were dissected, they were subject to RNA isolation. Dissected vessels were subject to 60 minutes of lysis buffer (from the RNeasy Kit) on ice (4°C) to permeabilize the microvascular cells followed by RNA isolation. The RNeasy Kit (Qiagen, Valencia, CA) protocol was applied to isolate RNA. After RNA isolation, samples were subject to vacuum centrifuge for 1 hr to increase RNA concentration. Once RNA was concentrated, it underwent reverse transcriptase to obtain the sample's cDNA for subsequent PCR. The protocols for semi-quantitative RT-PCR including reverse transcriptase procedures, LightCycler PCR procedures, and the primer sequences for eNOS and the internal control 18s rRNA were described previously in the materials and methods section of Chapter V. Other primers used for real-time RT-PCR in these experiments included primers specific for nNOS, the AT1 receptor, the AT2 receptor, and renin (Invitrogen, Carlsbad, CA). The nNOS primer sequences were (sense) 5'-ACTTACCAGCTCAAGGAC-3' and (antisense) 5'-GGCAGACCTGAGGTTC-3'. The nNOS primers amplified a 198 bp region within the
nNOS gene (1887-2084 bp). The AT1 receptor primer sequences were (sense) 5'-TAGCACTGGCTGACTTAT-3' and (antisense) 5'-AGGCAGGACTTCTATTG-3'. The AT1 receptor primers amplified a 205 bp region within the AT1 receptor gene (590-794 bp). The AT2 receptor primer sequences were (sense) 5'-TCTGACCTCTCTGGATG-3' and (antisense) 5'-CCCTTGGAGCCAAGTA-3'. The AT2 receptor primers amplified a 205 bp region within the AT2 receptor gene (984-1188 bp). The renin primer sequences were (sense) 5'-GTGCAGCCGTCTCTAC-3' and (antisense) 5'-CCGTGACCCTCTCCAAAC-3'. The renin receptor primers amplified a 188 bp region within the renin gene (392-579 bp). 18s rRNA was used as an internal reference. The expression of 18s rRNA is an effective internal reference because it does not undergo significant regulation in the postnatal immature kidney.

**Standard curves.** To generate eNOS, nNOS, AT1 receptor, AT2 receptor, renin and 18s rRNA cDNA standard curves, PCR products were separated by agarose gel electrophoresis. The products were purified from the gel using a gel extraction kit (Qiagen, Inc., Valencia, CA), quantitated by measuring absorbance at 260 nm on a DU-65 Spectrophotometer (Beckman-Coulter, Inc., Fullerton, CA), and used as DNA templates for quantifications using real-time RT-PCR. Products at varying concentrations were subject to amplification and subsequent real-time RT-PCR crossing points were used for generation of standard curves.

**Statistical analysis.** Experiments were repeated x6. All data are reported as means ± SE. Differences between age groups were determined by an unpaired t test. Values of p < 0.05 were considered statistically significant.
Tissue Analysis Techniques for NOS Activity Assay

Homogenization. Once microvessels were dissected, they were also subject to a NOS activity assay. Homogenization buffer was added to dissected vessels. The amount of homogenization buffer added to vessels was 4x (four times tissue to total volume). Homogenization buffer volume was approximately 1 ml. Homogenization buffer consisted of the following: EDTA 1mM, EGTA 1mM (6N NaOH was added as needed to dissolve EDTA and EGTA in dH₂O), sucrose 320 mM, Tris-HCl 50 mM, DTT 1 mM, PMSF 1mM, leupeptin 0.002 mM, pepstatin A 0.001 mM, mercaptoethanol 12 mM (Sigma, St. Louis, MO). The solution's final pH was adjusted to 7.4. Microvessels were homogenized via sonication using a Micro Ultrasonic Cell Disrupter (Kontes, Vineland, NJ) at 13 W.

NOS activity assay. 50 ul of the crude microvessel protein homogenate was used for the activity assay. 20 ul of assay buffer I was added to 50 ul of homogenate. Assay buffer I consisted of the following: Tris-HCl 25 mM, CaCl₂ 1.25 mM (pH adjusted to 7.2), NADPH 1mM, valine 10 mM, and proline 10 mM (Sigma, St. Louis, MO). 15 ul of cofactor solution was added to the sample. Cofactor solution consisted of the following: FAD 1.12 uM, FMN 1.12 uM, BH₄ 3.36 uM, calmodulin 0.112 uM, and DTT 1.12 uM (Sigma, St. Louis, MO). Samples were vortexed and incubated in a hot bath at 37°C for 15 minutes. 20 ul of assay buffer II was added to samples. Assay buffer II consisted of the following: Tris-HCl 25 mM, CaCl₂ 1.25 mM (pH adjusted to 7.2), NADPH 1 mM, 25.3 nmoles (4 mCi) of radio labeled ³H-arginine (Perkin-Elmer, Boston, MA) (4 ul of ³H-arginine per rxn). Non-labeled arginine was added to assay buffer II to bring the total arginine concentration up to 5 uM. After addition of assay buffer II, samples were
vortexed and placed in a hot bath at 37°C for 45 minutes. 400 ul of stop rxn buffer was added to samples following hot bath incubation. Samples were vortexed. Stop rxn buffer consisted of the following: EDTA 50 mM (6N NaOH was added as needed to dissolve the EDTA in dH2O) and HEPES 500 mM with pH adjusted to 5.5. 400 ul of Dowex hydrogen form resin (Sigma-50X8-400) (Sigma-Aldrich, St. Louis, MO) was added to samples. The Dowex resin was dissolved in a 50:50 suspension with dH2O. Samples were vortex for 5 minutes and centrifuged at 4000 rpm for 4 minutes. 500 ul of the resulting supernatant was combined with scintillation cocktail (Research Products International Corp., Mount Prospect, Ill.) in scintillation vials, mixed thoroughly, and subject to DPM count assessment using a LS 6500 Multi-Purpose Scintillation Counter (Beckman-Coulter, Inc., Fullerton, CA). For negative samples, all buffers and solutions, including fresh homogenization buffer and required NOS substrates, were added as described above except crude protein homogenate was excluded from the sample.

**Protein concentration assay.** To determine the total amount of protein in each sample, a fraction of each sample’s crude homogenate was used in a QuickStart Bradford Protein Assay (Bio-Rad, Hercules, CA). Protocol was followed as described in the Bradford Protein Assay. Samples of unknown protein concentration, samples with no protein (blanks), and standards with known protein concentration were used in the protein assay. Absorbance of each sample was taken on a spectrophotometer at 595 nm. Blank absorbance was subtracted form unknown samples and standards. Standards were used to generate a standard curve on which the quantity of protein was calculated for unknown samples.
Calculations. Fractional conversion of the conversion of L-arginine to L-citrulline was calculated by the following equation: 

\[
\frac{[\text{sample DPM} - \text{negative sample DPM}]}{[\text{total activity per sample without resin application} - \text{negative sample DPM}]} = \text{fractional conversion of L-citrulline.}
\]

The total activity per sample was obtained by adding fresh homogenization buffer (no protein), assay buffer I, cofactor solution, and assay buffer II and then assessing the amount of activity (in DPMs) in the solution using scintillation cocktail and the LS 6500 Multi-Purpose Scintillation Counter without the application of Dowex resin. Fractional conversion was multiplied by total arginine (\(^{3}\text{H}-\text{arginine plus non-labeled arginine}) in each sample to determine the amount of converted L-citrulline in each sample. The value for converted L-citrulline was expressed as moles of citrulline converted per minute of incubation during the activity assay per gram of total protein in each sample.

RESULTS

The successful staining of newborn renal microvessels with perfused blue-dyed polybeads is demonstrated in Fig. 26.

eNOS expression

The expression of eNOS mRNA increased in total preglomerular microvessels with maturation (Figs. 27 and 28). Lowest level of eNOS was found in the newborn while highest levels were observed in the adult. The expression of eNOS increased the greatest in the afferent arteriole during maturation. The lowest level of eNOS was
observed in the afferent arteriole of the newborn. The level of eNOS expression consistently increased in the afferent arteriole until reaching its greatest level of expression in the adult. The expression of eNOS did not experience much change in the interlobular arteriole during maturation. The expression of eNOS in the arcuate increased with maturation with a peak at 21 days. Arcuate arteriolar eNOS expression was the lowest in the newborn, while highest expression was observed in the more mature kidneys of the 21 day and adult.

Fig. 26. A newborn 500 um corticomedullary renal slice with polybead stained microvessels under stereomicroscope 16x magnification.
Fig. 27. Expression of eNOS mRNA in preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, adult aff vs. newborn and 7 day aff.

Fig. 28. Sum of eNOS mRNA expression in total (aff/int/arc) preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn vs. 21 day and adult.
The expression of eNOS in the vasa recta of the porcine kidney increased with maturation, similar to what was observed in preglomerular vessels (Fig. 29). The lowest level of eNOS expression was observed in the newborn while greatest expression was found in the adult. eNOS expression increased consistently in the 7 day and 21 day.

![Bar graph showing expression of eNOS mRNA in vasa recta in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn vs. 21 day and adult.]

**nNOS expression**

The expression of nNOS in total preglomerular vessels was greater at all immature ages than in the adult (Figs. 30 and 31). The expression of nNOS was found to increase from newborn to 7 day, then decrease from 7 day to 21 day before dipping to
Fig. 30. Expression of nNOS mRNA in preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn, 7 day, and 21 day aff, int, and arc vs. adult aff, int, and arc. #P < 0.05, 7 day aff and int vs. newborn and 21 day aff and int.

Fig 31. Sum of nNOS mRNA expression in total (aff/int/arc) preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, vs. adult. #P < 0.05. 7 day vs. newborn and 21 day.

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the lowest expression found in the adult. Highest nNOS expression was observed at 7 days. The expression of nNOS in specific vessels showed that expression changes in the afferent arteriole were minimal, while the greatest changes occurred in the interlobular and arcuate arterioles. The interlobular and arcuate arterioles both showed highest expression of nNOS at 7 days, while the adult displayed extremely low nNOS expression in all vessels studied.

The expression of nNOS in the porcine vasa recta was lowest, and relatively equivalent, in the newborn and 7 day kidneys, and increased with age to the highest levels in the 21 day and adult (Fig. 32). The expression of nNOS peaked to its highest

![Graph showing expression of nNOS mRNA in vasa recta in pigs of different ages.](image)

Fig. 32. Expression of nNOS mRNA in vasa recta in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn and 7 day vs. adult.
level in the 21 day kidney before dropping off slightly in the adult. The lowest level of expression was observed in the newborn.

**Renin expression**

The expression of renin in total preglomerular vessels was similar to that observed for nNOS (Figs. 33 and 34). Renin expression was highest in the newborn and 7 day and then decreased with age reaching its lowest expressional levels in the 21 day and adult. Renin expression was highest in the 7 day preglomerular vessels and lowest in the adult. Renin expression moderately changed in the afferent arteriole during maturation with highest renin levels found in the 7 day kidney followed by the newborn and 21 day with very low levels observed in the afferent arteriole of the adult. The

![Graph](image)

Fig. 33. Expression of renin mRNA in preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn and 7 day aff and int vs. 21 day and adult aff and int.

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The greatest change in renin expression occurred in the interlobular arteriole. Both the newborn and 7 day kidneys experienced very high renin expression in the interlobular arterioles before abruptly dropping to very low levels, which were found in the 21 day and particularly the adult. Low levels of renin expression were observed in the arcuate arterioles of porcine of all ages studied.

AT1 receptor expression

The AT1 receptor showed an expressional increase with maturation with a spike at 7 days (Figs. 35 and 36). AT1 receptor expression was lowest in the newborn and highest in the 7 day and adult kidneys. The greatest expressional change occurred in the afferent arterioles. The afferent arterioles followed the same pattern as did total
Fig. 35. Expression of AT1 receptor mRNA in preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, 7 day and adult aff vs. all vessels all ages.

Fig. 36. Sum of AT1 receptor mRNA expression in total (aff/int/arc) preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn and 21 day vs. 7 day and adult.
preglomerular expression, with lowest levels found in the newborn and highest levels found in the 7 day and adult kidney. There was slight fluctuation of AT1 receptor expression in the interlobular arteriole with highest levels found in the 7 day old kidney, while expression in the arcuate arterioles remained fairly constant at all ages.

**AT2 receptor expression**

The AT2 receptor showed expression that decreased with age with a substantial dip at 7 days in total preglomerular vessels (Figs. 37 and 38). AT2 receptor expression was by far highest in the newborn and lowest in the adult. AT2 receptor levels in the adult were near minimal detection limits. Fluctuation of AT2 receptor expression was apparent in all vessels between all ages, but particularly in the afferent arteriole. The afferent arteriole of the 7 day kidney had the greatest level of expression. The afferent,

![Fig. 37. Expression of AT2 receptor mRNA in preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn and 21 day aff, int, and arc vs. 7 day and adult aff, int, and arc.](image-url)
interlobular and arcuate arterioles all peaked in AT2 receptor expression in the newborn. All vessels studied showed lowest expression in the adult.

![Graph showing AT2 receptor mRNA expression](image_url)

**Fig. 38.** Sum of AT2 receptor mRNA expression in total (aff/int/arc) preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, vs. newborn and 21 day. #P < 0.05, 7 day vs. newborn, 21 day, and adult.

**NOS activity assay**

In the newborn, the production of NO, as measured by L-citrulline production and normalized by amount of total protein, was greatest in the interlobular arteriole followed by the arcuate and afferent, respectively (Fig. 39). NO production in the interlobular arteriole was over twice as high as that in the afferent arteriole in the newborn. As the kidney matured, there was a decrease in NO production in the afferent arteriole.
Fig. 39. Conversion of L-arginine to L-citrulline and subsequent production of NO as measured by NOS catalytic activity assay in pregglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. *P < 0.05, newborn aff vs. 21 day aff, int, arc; newborn aff vs. adult aff, int; #P < 0.05, newborn int vs. 7 day aff; newborn int vs. 21 day aff, int, arc; newborn int vs. adult aff, int, arc; †P < 0.05, newborn arc vs. 21 day aff, int, arc; newborn arc vs. adult aff, int.; §P < 0.05, 7 day aff vs. newborn int.; 7 day aff vs. 21 day aff, int, arc; 7 day aff vs. adult aff, int; &P < 0.05, 7 day vs. 21 day aff, int, arc; ‡P < 0.05, 7 day vs. 21 day aff, int, arc; 7 day vs. adult aff, int.

and interlobular arterioles, as found in the 7 day old kidney, however, NO production increased in the arcuate arteriole. In the 7 day old, NO production was highest in the arcuate arteriole, followed by the interlobular and afferent arterioles. After 7 days, NO production dramatically dropped off with kidney maturation. In the 21 day pregglomerular vessels, levels of NO production were nearly equivalent in all vessels with the afferent arteriole producing just slightly lower levels of NO as compared to the interlobular and arcuate arterioles. The amount of NO production seemed to level off at 21 days and adulthood as the adult exhibited NO production that was comparable to that found in the 21 day old. In the adult, afferent and interlobular arterioles produced
relatively the same amounts of NO while the arcuate arteriole produced slightly more NO.

The production of NO, as measured by L-citrulline production, was greater in all preglomerular vessels in the newborn as compared to the adult (Fig. 40). The afferent arteriole of the newborn produced over twice as much NO as the adult afferent arteriole. The interlobular arteriole of the newborn produced five times as much NO as the adult interlobular arteriole. The arcuate arteriole of the newborn produced three times as much NO as the adult arcuate arteriole.

The production of NO, as measured by L-citrulline formation, in total preglomerular vessels decreased with age (Fig. 41). NO production was highest in the newborn, decreased slightly in the 7 day old, and was significantly reduced in the 21
day and adult preglomerular vessels. Lowest levels of NO production were observed in the 21 day old, which was just slightly lower than NO production in the adult.

![Graph showing conversion of L-arginine to L-citrulline and subsequent production of NO as measured by NOS catalytic activity assay in total preglomerular microvessels in porcine ages newborn, 7, 21 day old and adult. N=6. †P < 0.05, newborn vs. 21 day and adult; *P < 0.05, 7 day vs. 21 day and adult.]

DISCUSSION

In the immature kidney, NO functions to provide counterbalancing vasodilation against the vasoconstriction of the highly activated RAS (124, 132). Intrarenal infusion of the NOS inhibitor, L-NAME, in both whole animal (piglets) (132), and isolated perfused kidneys (rabbits) (124), produces greater renal hemodynamic responses in the
immature kidney than in the adult. Such studies indicate that the renal hemodynamics of
the young is much more dependent on NO derived from NOS than the adult in order to
maintain normal physiological kidney function. However, quantification and
comparison of NOS isoform expression and function in immature and mature
preglomerular resistance vasculature has yet to be investigated.

Previous results from our lab indicate expression levels of eNOS mRNA in
whole kidney models are comparable between immature and mature kidneys with an
exception of an expression dip at 7 days post birth (129). A similar eNOS
expression pattern was confirmed by Han et al. (53) in studies on the developing rat
kidney. A difference in cortical and medullary eNOS expression was observed in our
lab where we found the renal cortex contains more eNOS than the medulla in the
immature whole kidney while the medulla contains more eNOS in the adult (129).
Interestingly, in this study, whole kidney trends of eNOS mRNA expression were not
observed in the preglomerular vessels of the developing porcine kidney. Instead, total
preglomerular eNOS expression was lowest at birth and progressively increased to its
highest levels in the adult. Reports from other labs using in situ hybridization have
localized eNOS expression to much of the developing renal vasculature including the
glomerular resistance vasculature (81, 83, 132, 141). In this study, eNOS was localized
to the afferent, interlobular, and arcuate arterioles. The afferent arteriole experienced the
greatest increase in eNOS expression with maturation. The expression of eNOS in the
afferent nearly tripled between the newborn and adult. The expression of eNOS in the
interlobular arteriole approximately stayed the same during maturation. The expression
of eNOS in the arcuate doubled during maturation. Although in whole kidney models
eNOS expression maintained a fairly constant level of expression throughout maturation from birth to adulthood, with the exception at 7 days post birth, the preglomerular resistance vessels showed a completely different novel developmentally regulated pattern of expression.

The lowest levels of eNOS expression at birth may indicate the isoform plays only a slight role in the newborn's renal hemodynamics, but a much larger role in the adult kidney. There is a significant eNOS expressional increase in the afferent arteriole where eNOS expression triples with maturation from neonate to adult. It appears through interpretation of expressional data, that eNOS may play a more important role in the adult vasculature than in the newborns'. It is of worth to note the increased expression of eNOS in the arcuate arteriole during maturation. The arcuate arteriole of the adult contained more eNOS expression than did that of the newborn. The data from our study agrees with other studies that have found arcuate arteriole endothelial cells strongly express eNOS from gestation throughout maturation into adult stages (78). eNOS in the adult arcuate arteriole may play a more pronounced role in RBF and GFR than it was previously thought.

While localization experiments in the piglet, confirmed by Fischer et al. (37), provide evidence that vascular nNOS distribution and expression in the developing kidney differs from that in the adult, the results of Fischer et al. indicates the absence of nNOS in the preglomerular resistance arterioles of the developing kidney. Other studies have led to variable results indicating both the presence and absence of nNOS in either the perivascular nerves or endothelium of the afferent arterioles, interlobular arterioles, and medullary vasa recta (7, 81, 83, 126). Studies by Mattson et al. identified both
nNOS and eNOS isoforms in the preglomerular vessels of the adult rat kidney including the arcuate, interlobular, and afferent arterioles, a finding that supports similar observations from other laboratories (7, 37, 141).

In this study, the expression of nNOS was observed in all vascular segments studied including the afferent, interlobular, and arcuate arterioles. Notably, the expressional pattern of nNOS was different than that for eNOS. The expression of nNOS was statistically higher at all immature ages in total preglomerular vessels as compared to the adult. Expression of nNOS was highest in the 7 day kidney, which was statistically higher than all other ages studied. In the interlobular and arcuate arterioles, the expression of nNOS was considerably higher than in the adult.

While our studies have indicated the presence of nNOS in the preglomerular vessels, the exact cellular location of nNOS mRNA is not known rather present in the perivascular nerves, vascular smooth muscle cells, or in the endothelial cells themselves. The perivascular nerves innervate the smooth muscle cells that are a part of the preglomerular resistance vessels. Kavdia et al. showed that NO derived through nNOS in the perivascular region can be a significant contributor to smooth muscle NO and thus would significantly affect available smooth muscle NO (76). The exact cellular location of nNOS within the preglomerular vessels requires further exploration.

In previous studies by our lab, nNOS expression was greatest in the whole kidney immediately after birth and decreased in expression with maturation to the lowest levels, which was observed in the adult, suggesting the role of nNOS was enhanced in the postnatal kidney (128). The elevation of nNOS expression seen in the preglomerular resistance vessels in this study suggests the augmented role of NO in the

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immature kidney is in part a function of developmentally regulated nNOS expression. The NOS isoform expressional results from this study indicate that during the postnatal period when the vasodilatory role of NOS is increased, NO may be predominately supplied by nNOS, rather than eNOS, as illustrated by the high nNOS expressional levels compared to the low levels observed for eNOS. The results in this study suggest nNOS serves as the main contributor of NO in the preglomerular vasculature of the immature kidney. As eNOS expression, initially low in the newborn, increases with maturation to adult levels, the need for nNOS in the resistance vasculature diminishes and thus nNOS expression decreases.

The expressional pattern for eNOS in the vasa recta was very similar to that found in the preglomerular resistance vessels. The expression of eNOS mRNA increased progressively with maturation with the newborn exhibiting the lowest level of expression and the adult expressing the highest level. The results here agree with other studies that found eNOS expression in the vasa recta gradually increased in the renal medulla after birth (78). The results of nNOS expression in the vasa recta were very similar to that observed for eNOS. The expression of nNOS increased with maturation with lowest levels being found in the newborn and highest levels found in the more mature animals. The only exception between eNOS and nNOS vasa recta expression was nNOS mRNA levels were slightly higher in the 21 day kidney as compared to the adult kidney while eNOS mRNA was highest in the adult. The significance for the up-regulation of either isoform in the vasa recta is not clear. As the mammalian kidney continues to mature, less sodium is reabsorbed and is therefore excreted by way of the tubules and collecting duct. NO derived from NOS within the vasa recta may serve two
purposes. One possibility may involve blood flow through the vasa recta. Increased NOS may in turn lead to increased NO levels in the vasa recta thus causing enhanced vasodilation. The increase in vessel luminal diameter would increase the blood flow through the vasa recta. The increase in blood flow in the vasa recta would increase hydrostatic pressure and thus reduce the amount of filtrate that is reabsorbed by the vasa recta causing an increase an excretion of ions such as sodium. Another way NO may influence reabsorption within the vasa recta is through a paracrine fashion. NO generated within the vasa recta may permeabilize the capillaries and enter the tubules and directly inhibit antiporters that function to transport and reabsorbed filtrate from the tubule lumen back into the bloodstream. At this point, the significance of increasing either vasa recta eNOS or nNOS expression with maturation is unknown.

Renin mRNA expression was high in preglomerular vessels in the newborn and 7 day kidney, but dropped considerably at 21 days and adult. Renin expression in the newborn and 7 day preglomerular resistance vessels was three and four fold, respectively, greater than in the adult. Expression of renin in the newborn and 7 day old was spread throughout both the afferent and interlobular arterioles, while the arcuate arteriole contained only very small amounts. Upon maturation, high renin expression receded to the afferent arteriole in the 21 day old before declining significantly throughout all preglomerular resistance vessels in the adult. The data from this study agrees with the data obtained from Gomez et al., in which it was found in rats, that renin expression is high throughout the preglomerular resistance vasculature, including the afferent and interlobular, of the immature kidney but expression declines and recedes back to the afferent arteriole with maturation (44).
Bachmann et al. demonstrated that changes in NOS and NOS gene expression parallel changes in renin and renin gene expression (9, 42). Studies directed at defining the type of NOS involved in renin changes have pointed to nNOS, with no consistent correlation with eNOS nor iNOS (42). The expressional results from this study would further suggest an interaction between nNOS and renin expression and activity. The developmental expressional pattern of nNOS and renin are almost identical. Both nNOS and renin expression is high in the newborn, peaks in the 7 day old, drops drastically in the 21 day old, and bottoms out in the adult. Both nNOS and renin are heavily expressed in the afferent and interlobular arteriole. The only difference between localization and abundance of mRNA transcription between the two genes is that nNOS is expressed in greater abundance than renin in the arcuate arteriole.

In adult models, NO has been found to influence renin secretion. NO inhibits cAMP degradation thus causing more renin to be secreted. However, NO mechanisms that alter intracellular calcium and activation of protein kinase C inhibit renin secretion (86). Although no direct correlation can be drawn between nNOS and renin expression or secretion in this study, the results here indicate nNOS and renin are both developmentally regulated and in a near identical pattern. It may be nNOS is the crucial NOS isoform in the immature preglomerular vasculature that provides defense against the enhanced vascular resistance found in the neonate kidney. Progressively, the kidney and vasculature matures, AII levels drop, and resistance declines, meanwhile nNOS levels decrease and eNOS expression increases and becomes the critical NOS isoform in the adult renal vasculature.
The sensitivity and reactivity to All is influenced by fluctuations in expression of the receptors that mediate the actions of All. In porcine, the effects of All are mediated through two receptors, the AT1 and AT2 receptors. When activated, it is believed both receptors activate G-protein associated second messengers, with the AT2 receptor activating phosphotyrosine phosphatases resulting in the inactivation of MAPKs (73). It is accepted that AT2 receptors are the abundant receptor type in the developing fetus and decrease in expression with birth and maturation. On the other hand, the AT1 receptor is minimally expressed in the fetus and newborn but increases with maturation to be by far the most abundant receptor subtype in the adult kidney. In fetal rat kidneys, 80% of All receptors are the AT2 subtype (73). It is believed that vasoconstriction, sodium reabsorption, and growth promotion occur via All through the AT1 receptor, while the AT2 receptor mediates vasodilation, natriuresis, and growth inhibition (73). Since all the components of the RAS are present in the developing embryo and are found in the kidney before the onset of fetal urine production, it is possible that the renal RAS may regulate growth and differentiation of developing nephrons in the fetus and neonate (73). Once kidney structures are completely formed, the RAS may then switch to a more primary role in regulating renal hemodynamics and sodium transport (73).

Both the AT1 and AT2 receptors exhibited developmentally regulated patterns in this study. Overall, AT1 receptor expression in the preglomerular vasculature was lowest at birth and progressively increased with maturation to its highest levels in the adult. The AT2 receptor exhibited highest expression in the immature preglomerular vessels and decreased to minute expression in the adult. Interestingly, the AT1 receptor
exhibited a spike in expression at 7 days while the AT2 receptor experienced an
expressional dip, possibly indicating a compensatory mechanism between the receptor.
Both nNOS and renin expression was also highest at 7 days. It appears that there is an
event, or possible transition of sorts, that occurs in the renal preglomerular vasculature
at 7 days as indicated by the results of this study. Intrinsic mechanisms within the
kidney may lead to neonatal changes during birth that effects mean arterial blood
pressure and alters renal blood flow and glomerular filtration rate. Another change may
occur at 7 days that allows renal hemodynamics to switch from the newborn state to an
immature condition that much more resembles adult renal proficiencies.

Other studies have found interactions between the expression of renin, All
formation and the All receptor subtype expression. Renal plasma All inhibits AT2
receptor expression (20). However, this is contradictive in the fetus and neonate where
both AT2 receptor and All plasma levels remain high. When the AT1 receptor is
activated, renin secretion is inhibited (20). The up-regulation of expression of the AT1
receptor that occurs in the maturing kidney, particularly at 7 days, may be one
mechanism used by the renal vasculature to decrease highly activated renal All. Studies
by other labs have demonstrated up-regulation of the AT2 receptor down-regulates the
AT1 receptor (20, 25, 44, 86). Another contributing mechanism to neonate renal
hemodynamics may be that AT1 receptor expression, maintaining relatively low levels
in the fetus and neonate, gradually increases with maturation as the inhibitory effects of
the AT2 receptor wear off as expression of the AT2 receptor diminishes with age.

In this study, both the AT1 and AT2 receptors were expressed throughout the
preglomerular vasculature. However, expression of the AT1 receptor was greatest in the
afferent arterioles indicating an enhanced role for the AT1 receptor in the afferent arteriole as compared to the interlobular and arcuate arterioles. The receptor findings in this study agree with previous data that indicates that AT2 receptor is more important in the immature kidney than the adult while the role of the AT1 receptor increases with maturation. Nevertheless, it appears an alteration in expression of both receptors is required for events that occur in the 7 day old preglomerular vasculature. The exact relationship between AT1 receptor, AT2 receptor, and NOS expression (particularly nNOS expression) is unknown. The results in this study indicate AII receptors, NOS and renin are all developmentally regulated in similar fashion with expression mirroring each other in the immature preglomerular resistance vessels. The RAS and nNOS isoform may possibly not only interact with each other to influence their expression, translation and physiological effects, but may as well be regulated by the same developmental factors.

Studies have found that increased NOS expression can influence NOS activity (26, 29, 59). Since protein expression does not always correlate with enzyme activity and NO production (59), it is important to observe actual NO production to accurately access the NO function in newborn renal hemodynamics. The NOS activity assay used in this study clearly identified an upregulation of NO production within the preglomerular resistance vessels of the immature kidney. NO activity was three to four times higher in the newborn and 7 day piglet as compared to the more mature 21 day piglet and adult. The NOS enzymatic activity assay data provides further evidence that the role of NO is enhanced in the neonate.
In 1993, Solhaug et al. (131) showed that NO modulates renal hemodynamics in the newborn piglet and that NO plays a much more important role in the renal hemodynamics of the immature kidney as compared to the adult kidney. Since that time, many studies have been designed to investigate the role of NO production in physiological adult renal hemodynamics, modulating All vasoconstriction, and modulating tubuloglomerular feedback. There have not been any studies designed to investigate the role of NO in the vasculature of the developing kidney, particularly in the preglomerular resistance vessels. In the adult preglomerular vasculature, Patzak et al. identified an interaction between All and NO release in afferent arterioles of the rat. Patzak et al. found that vasoconstrictor responses to All were enhanced in eNOS knockout mice and that All upregulates NO production via the AT1 receptor (104, 105). Thorup et al. found that inhibition of the AT1 receptor inhibited All stimulated NO production (135). An interaction between All and NO has been identified in the adult kidney. However, to day, the exact relationship between All and NO in the newborn kidney has not yet been fully investigated.

At the same time that the RAS is upregulated in the newborn’s preglomerular resistance vasculature, NO levels are also upregulated. In this study, the newborn’s interlobular arteriole produced the most NO followed by the arcuate and the afferent. In the 7 day old, the arcuate produced the most NO followed by the interlobular and the afferent. In the more mature kidneys, NO production was significantly and consistently lower in all preglomerular vessels examined.

The increase in NO production in the interlobular and arcuate may suggest an augmented role of these vessels in the modulation and regulation of RVR, RBF and
GFR in individual vascular channels within the kidney. In the newborn piglet kidney, nephrogenesis and angiogenesis are not complete until week three. As a consequence, many afferent arterioles are not developmentally ready to function and control filtration rates through branching nephrons. This may shift glomerular hemodynamic regulation to vessels that develop quicker than the afferent arterioles, including the interlobular and arcuate arterioles. Serving as a control valve to distribute blood flow to developing afferent arterioles, the arcuate and interlobular arterioles would be the more prominent site of vascular control. An enhanced role of vascular control would mean the role of vaso-active agents would also be enhanced in these segments.

Renin expression was widespread throughout the interlobular and afferent arterioles of the postnatal kidney. The AT1 and AT2 receptors were also widespread throughout the preglomerular vessels as well, but the AT1 and AT2 receptor transcripts were in much more abundance in the afferent arteriole, possibly suggesting an increase in afferent AII sensitivity. Increased action of AII in the afferent arteriole, particularly afferents attached to glomeruli that are still developing, would result in decreased blood flow to these glomeruli thus lowering their individual GFR. This may be a mechanism by which the immature kidney routes blood flow to more developed nephrons. Juxtamedullary nephrons have been found to develop before superficial nephrons. While resistance remains high in developing afferent arterioles throughout the immature kidney, particularly to superficial nephrons, blood flow may be distributed to fully developed glomeruli, particularly juxtamedullary nephrons, within the kidney by modification of resistance offered in the interlobular and arcuate arterioles.
NO production was highest in the interlobular and arcuate arterioles of the newborn and 7 day old kidney suggesting an enhanced need for NO production in those vascular segments. The NOS enzyme whose expression was elevated in the interlobular and arcuate segments of the newborn and 7 day old was nNOS, further implicating nNOS as the critical source of NO that opposes enhanced All activity. As the kidney develops and finishes with nephrogenesis and angiogenesis, the renal vasculature reaches adulthood status. In the adult, RVR is lower, RBF and GFR are higher, RAS activity decreases, nNOS expression decreases, and eNOS expression increases, and thus the primary control site for single nephron filtration is in the resistance offered by the afferent arteriole, while the role of resistance in the interlobular and arcuate arterioles, once critical in the neonate, is much more attenuated and secondary to the afferent arteriole resistance offered in the adult.

The results from this study demonstrated distinctive postnatal developmental expressional patterns were observed for nNOS, eNOS, renin and the AT1 and AT2 receptors in the preglomerular resistance vessels, as well as the vasa recta. The enhanced role of NO during postnatal maturation maybe produced by developmentally regulated NOS expression, particularly nNOS, in the immature renal vasculature, primarily the resistance arterioles. The expression of renin and All receptors follow the same developmental pattern observed for the high renal vasculature resistance found in the immature preglomerular resistance vessels. The expression of RAS components follows the same pattern as nNOS expression in the immature preglomerular resistance vessels. NOS enzymatic activity, also high in the neonate’s preglomerular resistance
vessels, decreases with maturation in similarly to nNOS expression and expression of RAS components.

The results of this study would agree with the hypothesis that the enhanced role of NO serving as a critical vasodilator, counteracting highly activated constrictors such as AII during postnatal renal maturation, is produced by developmentally regulated NOS expression and function in the immature preglomerular resistance vessels. While it was found that NO production was significantly upregulated in the neonate, it may be the result of increases in nNOS expression in the preglomerular resistance vasculature. Furthermore, this study indicates that the interlobular and arcuate arterioles may play a more important role in neonatal preglomerular resistance, subsequent RBF and GFR, and possibly single nephron filtration as compared to its adult counterpart. However, more studies are needed to investigate the role of NO in the preglomerular resistance vasculature of the immature kidney with particular emphasis on mechanisms of NOS expression and activity, and the role of NO in counterbalancing the enhanced vasoconstriction of AII.
CHAPTER VII

STUDY 4: ANGIOTENSIN II REGULATES NITRIC OXIDE SYNTHASE EXPRESSION AND NO FUNCTION IN THE RENAL RESISTANCE VASCULATURE DURING PORCINE POSTNATAL MATURATION

INTRODUCTION

Ubiquitous production of NO has been shown to be involved in the regulation of multiple processes throughout the body. In particular, NO plays a crucial role in multiple processes within the kidney including modulation of RBF. As time goes on and our knowledge of NO in the vasculature increases, the role of NO and its importance in maintaining normal physiological homeostasis increases as well. Although structural and physical factors play a role in the change in RVR and RBF seen in the developing kidney, it is accepted that an increase in functional vasoactive agents is the major cause for the increase in the neonate’s RVR.

The primary site of renal vascular resistance in the newborn kidney occurs in the preglomerular microvessels. The main site for the reduction in vascular resistance is the afferent arterioles, which are directly adjacent to glomeruli. Fluctuations in reactivity within the afferent arteriole directly impacts RBF and GFR. Thus, a decrease in vascular resistance in the afferent arteriole would have a large influence on the rate of
filtration. Therefore, an interaction, or counterbalancing between AII vasoconstriction and NO vasodilation in the afferent arteriole would impact RBF and GFR the greatest.

Both eNOS and nNOS mRNA and protein are developmentally regulated during postnatal maturation. Furthermore, developmental patterns of eNOS and nNOS are different suggesting the isoforms have different regulatory factors and functional contributions in the postnatal kidney (128, 129). Whole kidney studies demonstrate nNOS expression is greatest in the newborn and progressively decreases with maturation (128). nNOS may serve as an more important vasodilator in the newborn than in the adult. However, all studies by Solhaug et al. used whole kidney models. The exact relationship between NO and AII in the newborn vasculature has not been examined. To fully understand the interaction between NO and AII, particularly in terms of counterbalancing AII vasoconstriction and influencing neonate renal hemodynamics, it would be ideal to observe the interaction between NO and AII in isolated microvessels of the newborn kidney and compare the results with that of the adult. To our knowledge, no studies have looked at this relationship directly between NO and AII in the neonate’s pre-glomerular vasculature.

Studies aimed at looking at the potential interaction between NO and AII have been conducted in adult models, primarily in rats and mice. In the adult, Patzak et al. showed the vasoconstrictor response to AII was enhanced in eNOS knockout mice (105). Patzak also showed that AII upregulated NO production via the AT1 receptor in the afferent arteriole of adult rats (104). Thorup et al. observed AT1 receptor inhibition inhibited AII stimulated NO production in the renal arteries of adult rodents (135). Hennington et al. found that acute AII infusion (110 minutes) increased eNOS mRNA
by 70% without changing eNOS protein levels, whereas chronic infusion of AII (10 days) increased eNOS protein by 90% without changing eNOS mRNA levels (108). All these experiments were conducted in the absence of changes in flow or perfusion pressure as to avoid input from other factors such as the myogenic response and shear stress. Clearly, there is an important interaction between NO and AII in the renal vasculature. However, the exact relationship has yet to be studied, or even identified, in the renal vasculature of the immature kidney.

The objective of this study was to determine the role of AII as a regulator of nNOS and eNOS expression and function of NO production in the immature and mature renal resistance arterioles, primarily the afferent arteriole. The hypothesis of the study is that in the immature renal resistance vasculature, NOS expression and function are regulated by AII through the AT1 and AT2 receptors.

MATERIALS AND METHODS

Microdissection of microvessels. The animals, animal preparation, age of animals, surgery, intrarenal perfusion of polybeads, and microdissection of specific renal vasculature for these experiments followed the same protocol as was discussed in the materials and methods section of Chapter IV. At the conclusion of the microvessel microdissection, the experiment followed the following protocols.

Tissue analysis techniques for mRNA studies. At the conclusion of the 1.5 hr dissection period, microvessels were centrifuged at 12,000 g for 2 min. Supernatant was discarded. Assay buffer was added to microvessels. The assay buffer consisted of dissecting
solution, 2 U/μl RnAsin, 5 mM DTT and either 0.1 uM All (Sigma) for All, 0.1 uM candesartan and 0.1 uM All for AT1X, 0.1 uM PD 123319 and 0.1 uM All for AT2X, or nothing for control. While in assay buffer, the microvessels were placed in a water bath at 37°C for 1 hr. At the conclusion of the 1 hr, lysis buffer (from the RNeasy Kit) was added to the microvessels and mRNA isolation was started. To begin mRNA isolation, microvessels were homogenized via sonication at 13 W using a Micro Ultrasonic Cell Disrupter (Kontes, Vineland, NJ). After homogenization, the RNeasy Kit (Qiagen, Valencia, CA) protocol was applied to isolate mRNA. After RNA isolation, samples were subject to vacuum centrifuge for 1 hr to increase RNA concentration. Once RNA was concentrated, it underwent reverse transcriptase procedures to obtain the sample’s cDNA. The protocols for semi-quantitative RT-PCR including reverse transcriptase procedures, LightCycler PCR procedures, primer sequences for eNOS, nNOS and the internal control 18s rRNA, standard curves, and statistical analysis were described previously in the materials and methods section of Chapters V and VI.

**Tissue Analysis Techniques for NOS Activity Assay**

*NOS activity assay.* At the conclusion of the 1.5 hr dissection period, microvessels were centrifuged at 12,000 g for 2 min. Supernatant was discarded. In a total volume of 55 μl, pre-made assay buffers were added which contained the following: assay buffer I (10 μl) - DTT 1 mM, PMSF 1 mM, leupeptin 0.002 mM, pepstatin A 0.001 mM, mercaptoethanol 12 mM (Sigma, St. Louis, MO); assay buffer II (20 μl) - Tris-HCl 25 mM, CaCl₂ 1.25 mM (pH adjusted to 7.2), NADPH 1 mM, valine 10 mM, and proline

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10 mM (Sigma, St. Louis, MO); assay buffer III (15 ul) - FAD 1.12 uM, FMN 1.12 uM, BH₄ 3.36 uM, calmodulin 0.112 uM, and DTT 1.12 uM (Sigma, St. Louis, MO). To bring the volume up to 55 ul, either water (for control and All samples), 0.1 uM candesartan for AT1X samples, or 0.1 uM PD 123319 for AT2X samples was added. Samples were vortexed and incubated in a hot bath at 37°C for 15 minutes. After incubation, either 5 ul of All, at a final concentration of 0.1 uM, or water was added to designated samples. 20 ul of assay buffer IV was added to all samples. Assay buffer IV consisted of the following: Tris-HCl 25 mM, CaCl₂ 1.25 mM (pH adjusted to 7.2), NADPH 1 mM, 25.3 nmoles (4 mCi) of radiolabeled ³H-arginine (Perkin-Elmer, Boston, MA) (4 ul of 3-H arginine per rxn). Non-labeled arginine was added to assay buffer IV to bring the total arginine concentration up to 5 uM. Samples were mixed by pipeting and placed in a hot bath at 37°C for 60 minutes. 400 ul of stop rxn buffer was added to samples following hot bath incubation. Samples were vortexed. Stop rxn buffer consisted of the following: EDTA 50 mM (6N NaOH was added as needed to dissolve the EDTA in dH₂O) and HEPES 500 mM with pH adjusted to 5.5. Samples were briefly centrifuged to pelletize microvessels and the pellet was removed and used in the protein concentration assay described later for analysis of total protein content. 400 ul of Dowex hydrogen form resin (Sigma-50X8-400) (Sigma-Aldrich, St. Louis, MO) was added to samples' remaining supernatant. The Dowex resin was dissolved in a 50:50 suspension with dH₂O. Samples were vortex for 5 minutes and centrifuged at 4000 rpm for 4 minutes. 500 ul of the resulting supernatant was combined with scintillation cocktail (Research Products International Corp., Mount Prospect, Ill.) in scintillation vials, mixed thoroughly, and subject to DPM count assessment using a LS
6500 Multi-Purpose Scintillation Counter (Beckman-Coulter, Inc., Fullerton, CA). For negative samples, all assay buffers and homogenization solutions (including required NOS substrates) were added as described above except microvessels were excluded from the sample.

Protein concentration assay. To determine the total amount of protein in each sample, the palletized microvessels removed from the NOS activity assay were used in a QuickStart Bradford Protein Assay (Bio-Rad, Hercules, CA). Pelletized microvessels were placed in 1 ml of Bradford Protein Assay solution and homogenized using a sonicator at 13 W. Sonic homogenization was done in increments to ensure adequate homogenization of microvessels. Microvessel samples remained on ice during homogenization. The entire resulting homogenate was subject to the Bradford Protein Assay protocol as described in the kit. Samples of unknown protein concentration, samples with no protein (blanks), and standards with known protein concentration were used in the protein assay. Absorbance of each sample was taken on a spectrophotometer at 595 nm. Blank absorbance was subtracted from unknown samples and standards. Standards were used to generate a standard curve on which the quantity of protein was calculated for unknown samples. For protein concentration calculations, absorbances of unknown protein samples were used to calculate protein concentration using the standard curve.

Calculations. Fractional conversion of the conversion of L-arginine to L-citrulline, total activity, and all other NOS activity assay calculations were followed as described previously in the materials and methods section in Chapter VI.
RESULTS

The successful staining of microvessels, by perfusion of blue-dyed polybeads, is illustrated in Figs. 42 and 43. In Figs. 42 and 43, a corticomedullary kidney slice from a 7 day old piglet is stained with polybeads and the microvessels, including the arcuate, interlobular, and afferent arterioles, plus the capillary bed of the glomerulus, are visible. As illustrated, vessel diameters were less than 5 μm.

Fig. 42. Magnification (50x) of a porcine newborn corticomedullary kidney slice stained with perfused blue-dyed polybeads. Microvessels stained by polybeads include the arcuate, interlobular and afferent arterioles, plus the glomerular capillary bed.
eNOS Expression

Control expression of eNOS mRNA in afferent arterioles was highest in the newborn, and lowest in the 7 day afferent arterioles (Fig. 44). The control value for 7 day was 56% lower than newborn, while 21 day and adult control values were 15% and 17% lower, respectively, than newborn control.

All administration resulted in a statistically insignificant reduction of eNOS expression at all ages (Fig. 44).

Blockade of the AT1 receptor cause the greatest attenuation in eNOS expression in the newborn and the least attenuation in the 7 day (Fig. 44). When the AT1 receptor was blocked with candesartan before application of AII in newborn afferent arterioles, eNOS expression was reduced 95%. When the 7 day AT1 receptor was blocked prior to
AII administration, eNOS mRNA levels were reduced by 64%. When the 21 day AT1 receptor was inhibited, eNOS expression was reduced by 76%. Blockade of the adult AT1 receptor resulted in a 68% reduction in eNOS expression.

Inhibition of the AT2 receptor caused the greatest eNOS expressional attenuation in the newborn, and the least in the 7 day (Fig. 44). When the AT2 receptor in newborns was blocked by PD 123319 prior to AII application, eNOS expression was reduced by 72%. When the 7 day AT2 receptor was blocked prior to AII administration, eNOS mRNA levels were reduced by 51%. AT2 receptor inhibition in the 21 day resulted in a 64% reduction in eNOS. Blockade of the adult AT2 receptor resulted in a 64% reduction in eNOS expression.

Fig. 44. The expression of eNOS mRNA in the afferent arterioles of porcine of varying developmental ages as measured by real-time RT-PCR. N=6. *P < 0.05, vs. control. †P < 0.05, vs. AII.
nNOS Expression

Control expression for nNOS mRNA was highest in the afferent arterioles of the newborn (Fig. 45). Control nNOS expression in the 7 day was 82% lower than newborn, while 21 day and adult control nNOS expression was 76% and 90% lower, respectively, than the newborn.

The newborn demonstrated higher nNOS expression during administration of All as compared to all other ages studied (Fig. 45). All administration alone caused statistically insignificant alterations in nNOS expression in the newborn, 7 day, 21 day, and adult.

While AT1 receptor inhibition resulted in significant attenuation in nNOS expression in all immature afferent arterioles, the greatest attenuation was observed in the newborn (Fig. 45). Inhibition of the newborn AT1 receptor prior to All

Fig. 45. The expression of nNOS mRNA in the afferent arterioles of porcine of varying developmental ages as measured by real-time RT-PCR. N=6. *P < 0.05, vs. control. †P < 0.05, vs. All. #P < 0.05, newborn control and All vs. all columns of all other ages.

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administration caused an 85% decrease in expression. 7 day AT1 receptor inhibition reduced nNOS expression by 79%. When the 21 day AT1 receptor was blocked, nNOS mRNA levels dropped by 72%. AT1 blockade in the adult caused a 9% increase in nNOS expression.

Blockade of AT2 receptors caused the greatest attenuation of nNOS expression in the newborn and 21 day, with little effect on 7 day and adult nNOS expression (Fig. 45). AT2 receptor inhibition prior to All administration in the newborn resulted in a 52% attenuation in nNOS expression. The blockade of AT2 receptors in the 7 day afferent arterioles resulted in only a slight 5% decline in nNOS expression. When the 21 day AT2 receptor was blocked, nNOS mRNA levels dropped by 65%. Blockade of the adult AT2 receptor before administration of All resulted in a 23% decrease in nNOS expression.

**NO Activity**

Measurement of NO production in afferent arterioles by NOS activity assay resulted in a newborn control value that was 25% greater than control values for the adult (Fig. 46). When given All, NO production in newborn and adult afferent arterioles increased 18% and 24%, respectively.

When the newborn afferent arterioles were given the AT1 receptor inhibitor candesartan prior to All administration, NO production was attenuated by 31% (Fig. 46). Blockade of the AT1 receptor in the adult afferent arterioles caused a 63% attenuation in NO production (Fig. 46).
When the newborn afferent arterioles were given the AT2 receptor inhibitor PD 123319 prior to All administration, NO production was attenuated by 44% (Fig. 46). Blockade of the adult AT2 receptor caused a 34% attenuation in NO production.

![Graph showing nitric oxide production](image)

Fig. 46. The production of nitric oxide (NO) in the afferent arterioles of porcine ages newborn and adult as measured by quantification of H\(^+\) labeled L-citrulline formation using the NOS activity assay. N=6. #P < 0.05, newborn control vs. adult control. *P < 0.05, vs. control. †P < 0.05, vs. All.

**DISCUSSION**

These studies show both the AT1 and AT2 receptors control mRNA expression of eNOS. At each age studied, from immature to mature, inhibition of either All receptor resulted in significant reductions in eNOS mRNA transcripts within afferent arterioles. To our knowledge, this is the first report that inhibition of the AT1 and AT2
receptors results in a decrease of eNOS expression in the immature afferent arterioles of swine. The control eNOS expression data matches the data provided by our lab in whole kidney models in which eNOS expression was relatively high in the newborn and adult with a dip in expression at 7 days (129). In studies on rats by Han et al., the same whole kidney eNOS expressional dip was observed at 7 days (53). There may be a transition in the undeveloped kidney from the immediate period after birth, in which maternal factors or fetal programming may carry over into the period immediately after birth, until a transition in the immature kidney occurs 7 days after birth. However, the significance of such a transition has not been identified.

Although statistically insignificant, there was a trend of down regulation observed with acute AII administration. It is essential to note that AII administration in these experiments was topical and lasted only for 60 minutes at 37°C. The 60 minute incubation period with AII did result in increases in NO production as measured by the NOS catalytic activity assay. Hennington et al. observed a 70% increase in eNOS mRNA after 110 minutes of AII infusion (58). However, in those experiments, there were no recorded changes in eNOS protein levels and NO activity was not recorded. Since the vasoactive component is the free radical NO molecule itself, the measurement of NO would be the crucial item to be observed in experiments involving microvessels. Studies have shown the NO serves as a negative-feedback regulator of eNOS expression (47, 143). Therefore, AII may serve to upregulate eNOS expression when levels of NO are low, however, once protein levels increase and/or NOS catalytic activity increases to produce more NO, the AII induced increase in eNOS expression would be attenuated to stop over NO production. As seen in numerous studies, NO can also serve as a
negative-feedback regulator of the NOS protein itself. Thus, the degree of expression does not always correlate with enzymatic activity because increased NO levels can result in inhibition of NOS expression and/or activity. In regard to the studies by Hennington et al., NO levels may not be enhanced for a long duration such as 110 minutes, thus the negative feedback that accompanies increase NO production would be absent and as a result, eNOS may have been upregulated by AII. Although no reports have been seen that specifically links NO to inhibiting nNOS expression, it would not be surprising if it were the case as well.

There is substantial evidence provided in the data obtained from the AT1 and AT2 receptor inhibition experiments to provide a link between the two receptors and eNOS expression. At each age, blockade of each receptor resulted in a significant decrease in eNOS expression. It was generally thought that the AT2 receptor caused vasodilation (4). However, the results of this study indicated at all ages, blockade of the AT1 receptor caused a greater attenuation of eNOS expression. Previous in vivo functional studies in whole kidney models by our lab have also indicated the role of the AT1 receptor in modulating the NOS response (132). Some studies have shown the AT2 receptor assumes a more important role when NOS is inhibited (4). Nevertheless, both receptors significantly influenced the mRNA expression of eNOS in this study.

Unlike eNOS expression, the mRNA expression of nNOS was only influenced by AII receptor inhibition in the immature kidneys. In control afferent arterioles, nNOS expression was the greatest in the newborn before dropping by 80% in the 7 day, and 21 day, and adult kidneys. The control expressional values of nNOS agreed with the whole kidney nNOS expressional data obtained previously by our lab in which the highest
levels of nNOS expression was found in the newborn while lowest levels were recorded in the adult. Even though overall expression of nNOS was relatively lower than eNOS expression in the preglomerular vasculature of the newborn, recent studies by Kavdia et al. indicate that a source of NO derived through nNOS in the perivascular region can be a significant contributor to smooth muscle NO, thus effecting its vasodilatory state (76).

Inhibition of the AT1 receptor resulted in significantly reduced nNOS expression in all immature kidneys, but not in the adult. Inhibition of the AT2 receptor resulted in attenuated nNOS expression only in the newborn and 21 day kidney. As was the case with eNOS, the AT1 and AT2 receptors play a role in the regulation of nNOS expression in the afferent arterioles of the immature kidney. However, unlike eNOS, neither the AT1 nor the AT2 receptor plays a role in the expression of nNOS in the adult. Furthermore, the expression of nNOS was augmented in the newborn, suggesting a more crucial role of nNOS in the newborn’s vasculature, and much more influenced by All receptor inhibition, particular inhibition of the AT1 receptor subtype. Blockade of the AT1 receptor in the newborn blocked 85% of the expression of nNOS. The results from this study agree with previous results from our lab that have indicated an enhanced role of nNOS in the developing kidney of the newborn as compared to the adult (128). The data obtained here also indicates that nNOS is heavily regulated by All through the AT1 and AT2 receptors in the newborn, but not in the adult. This may indicate that NO produced by nNOS may be an important influence in modulating newborn renal hemodynamics opposing the enhanced activity of the RAS.

The exact mechanism by which antagonist block either eNOS or nNOS expression is unknown. Both receptor subtypes have been localized to the surfaces of
endothelial cells and vascular smooth muscle cells (VSMC). Activation of the receptors on endothelial cells can promote the production of NO from presumably eNOS, nNOS expression may as well be effected. Generation of NO within VSMC from activated AII receptors on the VSMC would require the NOS enzyme to be present within the VSMC, thus requiring the transcription of NOS within the cell. There may as well be some sort of cross talk between AII receptors on endothelial or VSMC and the perivascular nerves that release nNOS mediated NO. In VSMC, the AT1 receptor activates at least two calcium mechanism as well as protein kinase C (PKC). The AT1 receptor is linked to a G-protein which can activate phospholipase C thus leading to formation of IP3 and subsequent calcium release from internal stores. The AT1 receptor has also been shown to open channels in the cell membrane to allow an influx of calcium to occur. Activated PKC and voltage-gated channels within the membrane can also effect calcium influx. It may well be the case that the same increase in intracellular calcium that marks the vasoconstrictor response to AII induces not only the NO production from NOS isoforms, but also the expression of NOS isoforms.

The exact mechanism by which the AII receptor subtypes influence NOS expression is not know. The 5'- flanking sequences of eNOS and nNOS have been obtained in other molecular studies (39). The nNOS 5'- flanking sequence contains a TATA box and two inverted CAAT boxes. Potential binding sites for transcription factors are found such as AP-2, transcriptional enhancer factor-1/M-CAT binding factor, CREB (cAMP response element binding protein), ATF (activating transcription factor), c-fos, Ets, NF-1 (nuclear factor 1), and nuclear factor kB (NF-kB) (39). The 5'- flanking genomic region of eNOS indicates that eNOS is 'TATA'-less and exhibits
proximal promoter elements consistent with a constitutively expressed gene in endothelial cells such as Sp1 and GATA motifs (156). The eNOS promoter contains consensus sequences for the binding of transcription factors AP-1, AP-2, NF-1, nuclear factor IL6, NF-kB, PEA3, and CREB (39). Upon AII receptor activation, the signaling cascade may lead to NOS transcription through the activation of such transcription factors.

As more data is obtained regarding NO in the preglomerular vasculature of either immature or mature kidneys, it seems the regulation of NOS activity is paramount in controlling NO production and that expression control is secondary. In our study here, topical administration of AII caused an increase in NO production in both the newborn and adult kidney. There was a 25% greater NO production in control newborn kidney as compare to the adult kidney. In the newborn, AT2 receptor blockade attenuated NO production greater than AT1 receptor blockade (44% and 31%, respectively). In the adult, the opposite occurred. AT1 receptor inhibition attenuated NO production greater than AT2 receptor inhibition (63% and 34%, respectively). The results are in agreement with the results found by Patzak at al. and Thorup et al. in adult rodents that AII through the AT1 receptor regulates NO production (104, 135). Furthermore, results from the data in this study may explain why in previous studies performed in our lab found that L-NAME caused alterations in newborn but not adult renal hemodynamics. The role of NO is enhanced in the newborn and that blockade of NOS activity would have an augmented impact on newborn renal hemodynamics.

In the newborn, control and AII alone NO production was in the range of 0.15-0.20 nanomoles of L-citrulline converted per minute per total nanograms of protein.
One mechanism through which NO elicits its vasodilatory effect on VSMC is through soluble guanylate cyclase (sGC). NO binds to the heme molecule in guanylate cyclase thus activating it causing an elevation in levels of cGMP. The effects of cGMP are mediated through the receptor proteins for cGMP. There are three major types of receptor proteins for cGMP: cGMP-binding phosphodiesterase, cGMP-dependent protein kinases, and cGMP-regulated ion channels. Even though cGMP kinases are the major receptor proteins expressed in vascular smooth muscle cells and play a large role in counterbalancing vasoconstriction with vasodilatation, contributions from phosphodiesterases and ion channels cannot be ignored (73). The reported NO concentration required for half-maximal activation of sGC is 1.7 nM (50). The range of NO production observed in this study is in the range of the required concentration needed to activate sGC. The reduced amount of NO produced from All receptor inhibition, particularly in the newborn, supports the hypothesis of this study that NOS function and NO production are regulated through the AT1 and AT2 receptors. The data from this study fits with the theory that NO counterbalances All in the immature kidney. When All activates All receptors, NO production goes up thus activating sGC causing a cascade of events that would counterbalance the All induced constriction of the afferent arteriole in the immature kidney. When the All receptors are inhibited, thus mimicking decreased levels of All, NO production is reduced and the effects on sGC activation are reduced.

The tight regulation of NOS and the production of NO occurs through multiple levels including transcription, spatial proximity near the intended target, enzyme covalent modifications, and allosteric regulation of the enzymes (82). Spatial proximity
near the intended target and enzyme covalent modifications seems to be the first line of 
regulation in terms of NO production by NOS enzymes. When NO is required quickly 
to oppose vasoconstriction, as would be the case in vivo in preglomerular resistance 
vessels, a fast working mechanism to turn on NO production would be required. In 
addition, the NO molecule has a very short half life of only 5-6 seconds and can be 
scavenged quickly in the vasculature by hemoglobin, albumin, and reactive oxygen 
species, so it is essential that NOS enzymes be in the most advantageous location 
possible which would be very near the intended target. Also, it’s vital the NOS enzyme 
be localized in the cell where its required substrates are readily available for NO 
production.

Most of eNOS in endothelial cells dock to the plasma membrane in a complex 
with caveolin-1, which serves to inactivate NO production. When endothelial cells are 
activated by increases in intracellular calcium, eNOS disassociates away from the 
caveolin complex and becomes active. The increase in calcium also promotes the 
interaction of eNOS to porin to further augment NO production (97). The early calcium 
dependent activation of eNOS is followed by a late phosphorylation dependent 
activation by protein kinase B/Akt and protein kinase A (18, 29, 41). Heat-shock protein 
90 binds eNOS and facilitates calmodulin dependent disruption of eNOS binding with 
caveolin and mediates the interaction between eNOS and protein kinase B/Akt (17, 38, 
49). Heat-shock protein 90 links eNOS to sGC in such cells. Efficient supply with 
substrate during this is ensured by localization of the arginine transporter cationic amino 
transporter (CAT1) in caveolae and its direct interaction with eNOS (93). NOSIP and 
NOSTRIN are two proteins that bind to eNOS and inactivate the enzyme.
In the perivascular nerve cells, the N-terminus of nNOS binds via PDZ-PDZ interaction to postsynaptic density proteins, PSD95 or PSD93, which further couple nNOS to N-methyl-D-aspartate (NMDA) receptors (16, 26). This location puts the nNOS protein in close proximity to calcium through NMDA glutamate induced calcium influx. The PSD95 proteins link nNOS to sGC thus allowing close proximity for NO to travel to activate sGC in such cells. Heat shock protein 90 interacts also with nNOS and may help facilitate calcium-calmodulin dependent nNOS activation. CAPON is a binding protein in neuronal cells that inhibits nNOS when it binds to the enzyme.

Besides protein-protein interactions, direct phosphorylation of the NOS enzymes themselves has been found to activate or inactivate the enzymes. Increased phosphorylation of the threonine residue at 495 and the serine residue at 633 has been shown to decrease NOS catalytic activity. Serine phosphorylation at residues 1177 and 1179 has been found to increase NO catalytic activity (88). Taken collectively, NO production and NOS regulation is a complex issue.

It is essential to ask what is the effect of NO on renin secretion. In some studies, NO has been found to both inhibit and enhance renin release (84, 86, 144). NO is known to inhibit cAMP degradation through cGMP mediated mechanisms. With the build up of cAMP, MLC phosphorylation decrease thus decreasing vasoconstriction and invoking more renin to be secreted into the vasculature (86). At the same time, calcium influx into the cell and the activation of PKC inhibits renin release (86). Overall, NOS blockade causes decreases in plasma renin activity and plasma angiotensin II while no change occurs in kidney tissue All with an increase in All effects on the renal
microvasculature (42). The effect of NO on renin secretion still requires further investigation.

The studies here demonstrate both AII receptor subtypes regulate of NOS expression and function in the neonate’s afferent arterioles. AT1 and AT2 receptor inhibition attenuated eNOS expression in afferent arterioles of kidneys all ages. AT1 and AT2 receptor inhibition attenuated nNOS expression only in the immature kidney’s afferent arteriole. In the immature afferent arterioles, AT1 receptor inhibition caused a greater attenuation of both eNOS and nNOS expression than did AT2 receptor inhibition. nNOS demonstrated increased expression in the afferent arteriole of the newborn as compared to the adult, further suggesting the role of nNOS is enhanced in the neonate’s preglomerular resistance vessels. NOS enzymatic activity was also attenuated by both AT1 and AT2 receptor inhibition in both newborn and adult afferents. AT2 receptor inhibition caused greater attenuation in NOS activity in the afferents of the newborn while AT1 receptor inhibition attenuated NOS activity to a greater extent in the adult. In all expressional studies, the AT1 receptor was shown to influence both eNOS or nNOS expression to a greater extent than the AT2 receptor. This may suggest differing roles between the two receptors during renal maturation where the AT1 receptor influences NOS expression to a greater extent in the immature afferent while the AT2 receptor’s role in the neonate’s afferent may be to modulate NOS enzymatic activity. The data provided here supports the hypothesis that in the immature renal resistance vasculature, primarily the afferent arteriole, NOS expression and function are regulated by AII via the AT1 and AT2 receptors.
CHAPTER VIII

CONCLUSIONS

While many studies have been directed towards studying the role of NOS isoforms in the adult renal vasculature and the subsequent effect NO has on the adult’s renal hemodynamics, to date, no labs have investigated the role of NOS isoforms in neonate renal hemodynamics. The renal homodynamic state of the neonate is quite different than that of the adult. Systemic blood pressure is lower in the newborn and leads to variable effects in the kidney. Autoregulation exists in the newborn kidney but is set at a lower perfusion pressure and is less efficient than in the adult. Tubuloglomerular feedback in the newborn has a lower maximal response and lower flow rate than in the adult, thus vasoactive feedback in the newborn is effected as well. Nephrogenesis and angiogenesis, complete in some species by birth, continues in the porcine kidney after birth. Developmental up and down regulation of various vasoconstrictors, vasodilators, and hormones also occurs in the neonate’s developing kidney. All these factors work in a complex, dynamic fashion to produce a renal hemodynamic state that is unique to the neonate. The unique renal hemodynamic state of the neonate predisposes the newborn to develop vasomotor ARF from adverse pathologies that further decrease the already low RBF found in the developing kidney, such as hypotension and hypoxia of perinatal asphyxia, or complications of prematurity. With this in mind, it is critical to gain an understanding into what are the main factors that control the renal hemodynamic state of the neonate.
Previous studies by our lab have indicated unspecific NOS inhibition with L-NAME in whole porcine kidneys causes an increase in RVR, with subsequent reductions in RBF and GFR in the immature, but not mature, kidney. This finding was the first to suggest NOS derived NO plays a more important role in the renal hemodynamics of the neonate than in the adult. Other studies found AII is the major factor that contributes to the enhanced RVR that is observed in the immature kidney. Our lab investigated the role of NO vasodilation providing counterbalancing to AII vasoconstriction in whole porcine kidneys and found that in the newborn, blockade of the AT1 receptor attenuated the response of L-NAME in the newborn, but not in the adult kidney. This finding was the first to suggest a developmental interaction between AII vasoconstriction and NO vasodilation. Our lab continued to study this phenomenon by studying the mRNA expression and protein translation of NOS isoforms in the kidney during development. Both expressional and translational studies indicated that in the immature whole kidney, nNOS and eNOS isoforms were developmentally regulated. Whole kidney expressional studies further suggested nNOS may be the major NOS isoform responsible for the enhanced role of NO in the immature kidney. These previous studies by our lab have indicated NO plays an enhanced role in the renal hemodynamics of the neonate as compared to the adult, and that NO works to buffer AII vasoconstriction, but the mechanism of developmental regulation, which isoform does what, the localization of specific NOS isoforms, and the exact relation of interaction between AII and NO were unknown and required further study.

Based on previous results and preliminary data, the overall hypothesis of this dissertation was generated, which was the enhanced vasodilatory role of NO in the
immature kidney is attributed to regulatory, expressional, and functional differences in eNOS and nNOS isoforms from the adult. The objective of the studies in this dissertation was to: (1) determine which NOS isoform regulates immature renal hemodynamics by using functional whole animal studies utilizing intrarenal infusion of NOS inhibitors; (2) continue characterization of NOS expression in specific immature renal microstructures utilizing LCM (glomerular eNOS expression); (3) characterize developmentally regulated expression and functional patterns of NOS isoforms, renin, and AT1 and AT2 receptors in the immature preglomerular resistance vasculature utilizing novel microdissection techniques; and (4) determine All regulation of NOS expression and function in the immature renal vasculature using AT1 and AT2 receptor inhibitors.

The first study in this dissertation was designed to compare the renal hemodynamic responses to the intrarenal infusion of the selective nNOS inhibitor L-SMTC with the intrarenal infusion of the non-selective NOS inhibitor L-NAME in both the immature kidney of the piglet and the mature kidney of the adult. The intrarenal arterial infusion of the nNOS-specific inhibitor, L-SMTC, in the immature piglet resulted in significant changes in RBF, GFR and RVR that were similar to those seen with L-NAME. However, infusion of L-SMTC in the adult did not alter renal hemodynamics or function. The piglet responses to nNOS inhibition were significantly greater than the adult pig responses to L-NAME. The responses to inhibition of the nNOS isoform seen in only the immature kidney in our experiments suggest a more important role for NO produced by nNOS during postnatal renal maturation. The findings from these results suggested nNOS was the major NOS isoform contributing to
the enhanced role of NO in the immature, but not mature, kidney. The findings supported the hypothesis of the first series of experiments that nNOS is the major isoform regulating renal hemodynamics in the immature, but not in the mature adult kidney. The conclusions of the first series of experiments were under basal, physiologic conditions, nNOS is an important regulator of renal hemodynamics and function in the immature kidney, but not in the adult, and may be the predominate vasoactive NOS isoform during postnatal renal maturation.

Our lab had previous started to identify expressional developmental regulation of NOS isoforms in specific kidney structures. The macula densa was studied and it was found nNOS, the major NOS isoform present within this structure, was developmentally regulated in the macula densa. The expression of nNOS was greatest in the newborn and progressively decreased with maturation to reach its lowest expressional level in the adult. nNOS in the macula densa plays a role in tubuloglomerular feedback. NO derived from macula densa nNOS contributes to the vasoactive function of the resistance arterioles, the afferent and efferent, but its role appears to be primarily to buffer the TGF response, primarily in the afferent arteriole. During the TGF response, AII levels are increased in the resistance arterioles and nNOS increases NO release from the macula densa to buffer AII vasoconstriction. When the macula densa senses high levels of NaCl, the TGF response of the macula densa cells do not increase the release of renin and macula densa nNOS production of NO is reduced. The tubuloglomerular feedback mechanism primarily works to insulate the kidney during changes in pressure, thus it appears NO derived from macula densa nNOS only plays a role in adjusting the

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filtration load into the tubules and does not play a role in normal continuous basal physiology of the renal vasculature during development.

At this point, the developmental regulation of NOS in the macula densa was established and it was time to investigate NOS in other renal microstructures during renal development. The second series of experiments in this dissertation were designed to do just that. The second series of experiments were aimed at investigating basal eNOS expression and eNOS regulation by AII in the glomeruli of the developing kidney. Furthermore, the second series of experiments also looked at the differences in eNOS expression in glomeruli located in the inner cortex, juxtamedullary glomeruli, as compared to the outer cortex, superficial glomeruli. Juxtamedullary glomeruli develop before superficial glomeruli during renal maturation so differences in eNOS expression were expected. Previous studies have determined nNOS is not present in the glomeruli so only eNOS was analyzed in the second series of experiments. The objective of the second series of experiments was to compare eNOS gene expression between superficial and juxtamedullary glomeruli during both basal conditions and AT1 receptor inhibition with candesartan using LCM to selectively harvest glomeruli from immature and mature porcine kidneys. The hypothesis of the second series of experiments was there are greater differences between superficial cortical and juxtamedullary glomerular eNOS expression in the immature kidney compared to the adult and that the AT1 receptor modulates expression of eNOS in the glomeruli of the newborn kidney, but not in the adult.

The data in the second series of experiments was able to show eNOS expression was much higher in the juxtamedullary glomeruli than superficial in the newborn.
Juxtamedullary glomerular eNOS expression is constant throughout maturation with the exception of an expressional decrease at 7 days. eNOS expression in superficial glomeruli is initially low in the newborn kidney, but increases in expression two weeks after birth. The expression of eNOS in either juxtamedullary or superficial glomeruli is regulated by AII via the AT1 receptor in the newborn, but not in the adult. The results of this study support the original hypothesis of this series of experiments. At this point, all studies, including whole kidney, macula densa, and glomeruli studies, exhibited developmental regulation of both eNOS and nNOS. Such findings support the theory that the enhanced role of NO as a critical vasodilator is produced by developmentally regulated nNOS and eNOS expression. Functional studies involving whole animals and NOS inhibitors in the first series of experiments indicated nNOS was the primary NOS isoform that influences neonate renal hemodynamics. Glomerular eNOS may contribute to the newborn’s renal hemodynamic state, particularly to counterbalance AII vasoconstriction, but the major site controlling the neonate’s renal hemodynamics is present in the resistance arterioles, particularly in the preglomerular microvessels.

The third series of experiments was aimed at comparing nNOS and eNOS expressional and NO functional differences between the preglomerular microvessels, including the afferent, interlobular, and arcuate arterioles, of the immature as compared to the mature kidney. Previous studies have identified the preglomerular microvessels as the primary site of RVR within the kidney. Therefore, vasoactive substances that effect RVR in the preglomerular microvessels would have the greatest impact on RBF and GFR within these vessels. The highly activated RAS has been shown to maintain the high RVR present in the immature preglomerular microvessels. Previous studies by our
lab have indicated NO produced within the renal microvasculature counterbalance AII vasoconstriction in the immature kidney. However, regulation and location of NOS within the immature renal vasculature was yet to be studied. The objective of the third series of experiments was to analyze mRNA expression of nNOS and eNOS along with NOS enzymatic catalytic activity in the preglomerular microvessels during renal maturation. The hypothesis of the third series of experiments was that the enhanced role of NO as a critical vasodilator, counteracting highly activated constrictors such as AII during postnatal renal maturation, is produced by developmentally regulated nNOS and eNOS expression and NO function in the immature renal vasculature, primarily in the resistance arterioles. Since it is believed there is an interactive counterbalancing between NO and the RAS, components of the RAS, including renin expression and AT1 and AT2 receptor expression, were also observed and compared.

The results from the first series of experiments in this dissertation indicated nNOS was the primary NOS isoform responsible for the enhanced role of NO in the immature renal vasculature. The mRNA expressional data from this third series of experiments further support the hypothesis that nNOS is the major NOS isoform effecting renal hemodynamics in the immature kidney. nNOS expression was greatest in all preglomerular microvessels of the newborn and 7 day old immature kidneys, but decreased progressively with maturation after spiking at 7 days. The greatest change in nNOS expression occurred in the interlobular and arcuate arterioles. Conversely, eNOS expression was lowest in the newborn, but slowly increased with maturation. The greatest increases in eNOS expression occurred in the afferent and arcuate arterioles. This further suggests nNOS is the crucial NOS isoform in the neonate’s renal
vasculature, while eNOS is the more critical NOS isoform in the adult kidney. The results from the NOS enzymatic catalytic activity assay showed NO production was the greatest in all preglomerular resistance microvessels of the newborn and 7 day old kidneys, and the lowest in the preglomerular resistance vessels of the older, more mature, 21 day old and adult kidneys. NO production was greatest in the newborn’s interlobular and arcuate arteioles. The results from the NOS enzymatic catalytic activity assay are further evidence that NO production within the renal vasculature is enhanced in the immature kidney as compared to the mature kidney. Thus, in the newborn when eNOS expression is low and nNOS expression is high, the production of NO is the greatest further implicating the role of nNOS in the immature renal resistance vasculature is augmented.

Previously, it was believed the afferent and interlobular arterioles were the main preglomerular microvessels that influence RBF and GFR. The results from this study indicate the arcuate arteriole may also play a crucial role in RBF and GFR in the neonate. Both nNOS and eNOS expression was also detected in the vasa recta of the developing kidney. However, both NOS isoforms were lowest in expression at birth and gradually increased with maturation to highest levels in the adult vasa recta. While not effecting RBF and GFR, NOS expression in the vasa recta is still of importance because dilation and constriction of vasa recta capillaries directly influence the reabsorption of substances from the tubules back into the blood in addition to the direct effect NO has on reabsorption activity.

Interestingly, the expression of renin paralleled the expression of nNOS in all preglomerular resistance vessels at all ages, particularly in the newborn and 7 day old
immature kidney. Renin expression was greatest in the newborn and spiked at 7 days while lowest expression was observed in the adult kidney. Greatest maturational change in renin expression occurred in the afferent and interlobular arterioles. AT1 receptor expression was lowest at birth and progressively increased with maturation with the exception of an expressional spike at 7 days. Expression of the AT2 receptor was high in the newborn and progressively decreased with maturation, with greatest expression in the newborn and 21 day. These results suggest the AT2 receptor may play an enhanced role in the newborn while the AT1 receptor plays a more crucial role in the adult. However, at 7 days, AT2 receptor expression decreases significantly before increasing again at 14 days. During that period of AT2 receptor downregulation, the AT1 receptor is upregulated suggesting a cross-talk mechanisms between both receptor subtypes. Both receptors were expressed heavily in the afferent and interlobular arterioles, however, the AT2 receptor was much more abundantly expressed in the arcuate arteriole than was the AT1 receptor. These results suggest nNOS, eNOS, renin, AT1 receptor, and AT2 receptor are all developmentally regulated in the immature porcine kidney. Furthermore, these studies suggest a developmental interaction between renin expression and nNOS expression in preglomerular microvessels. The actions of AII may be mediated through primarily the AT2 receptor in the newborn and the AT1 receptor in the adult while both receptors coordinate to mediate AII responses in the 7 day old kidney. When nNOS expression is high and eNOS expression is low, NO production is the greatest in the neonate further suggesting nNOS is the major NOS isoform effecting renal hemodynamics in the neonate. Renin expression and AII receptor expression parallel nNOS expression to a certain degree suggesting an
interaction between the RAS and nNOS during development. The results here are in agreement with the hypothesis of the third series of experiments.

The third series of experiments was able to show developmental regulation mRNA expression of NOS isoforms and NO production in the preglomerular microvessels with considerable similarities between expression of nNOS and components of the RAS. The expression of renin and AT receptors follow the developmental pattern associated with high renal vasculature resistance found in the immature kidney. The components of RAS also follows the regulation of NOS, particularly nNOS, in the immature kidney. NOS enzymatic activity in the preglomerular resistance vessels was high in the newborn and decreased with maturation. The enhanced role of NO during postnatal maturation maybe produced by developmentally regulated NOS expression, particularly nNOS, in the immature renal vasculature, primarily the resistance arterioles.

The focus of the forth series of experiments was to investigate the role of NO vasodilation regulation by AII since it is believed NO vasodilation counterbalances AII vasoconstriction. The objective of the forth series of experiments was to determine the role of AII as a regulator of nNOS and eNOS expression and function of NO production in the immature and mature renal resistance arterioles, primarily the afferent arteriole. The hypothesis of the forth series of experiments was that in the immature renal resistance vasculature, NOS expression and function are regulated by AII through the AT1 and AT2 receptors.

Inhibition of the AT1 receptor by candesartan resulted in reduction of eNOS expression in afferent arterioles of kidneys of all ages studied, both immature and
mature. Attenuation of eNOS expression was greatest in the newborn where eNOS expression was reduced by 96%. Attenuation of eNOS expression was comparable in afferent arterioles of kidneys of all other ages studied ranging from 64% to 76% reduction in eNOS expression. Inhibition of the AT2 receptor by PD 123319 also resulted in a decrease of eNOS in afferent arterioles of kidneys of all ages. Again, the greatest reduction in eNOS expression was witnessed in the newborn’s afferent arteriole with 72% reduction in eNOS expression with AT2 receptor inhibition. Inhibition of the AT2 receptor in afferent arterioles of kidneys at other stages of maturation resulted in reduction of eNOS expression that ranged from 51% to 64%.

The results here demonstrated AT1 and AT2 receptor inhibition attenuated eNOS expression in afferent arterioles of kidneys all ages. AT1 and AT2 receptor inhibition attenuated nNOS expression only in the immature kidney’s afferent arteriole. In the immature afferent arterioles, AT1 receptor inhibition caused a greater attenuation of both eNOS and nNOS expression than did AT2 receptor inhibition. NOS enzymatic activity was attenuated by both AT1 and AT2 receptor inhibition in both newborn and adult afferents. AT2 receptor inhibition caused greater attenuation NOS activity in the afferents of the newborn than adult.

Interestingly, even when the AT1 receptor was low in expression, such as in the newborn and 21 day old afferent arterioles, as observed in the third series of experiments, inhibition of the AT1 receptor still resulted in significant reduction in eNOS expression. Similar, even when the AT2 receptor was observed to be reduced in expression, as seen in the 7 day old and adult afferent arterioles, as observed in the third
series of experiments, inhibition of the AT2 receptor still resulted in significant reduction in eNOS expression.

Earlier experiments in this dissertation have suggested nNOS is the crucial NOS isoform operating within the neonate's preglomerular resistance vessels working to oppose enhanced AII activity. It would be expected that AII would have an influence on nNOS expression in the afferent arterioles of the neonate. The expression of nNOS was attenuated in the afferent arterioles to a greater extent by inhibition of the AT1 receptor than by inhibition of the AT2 receptor. AT1 receptor inhibition attenuated nNOS expression the greatest in the newborn reducing nNOS expression by 85%. AT1 receptor inhibition also caused significant nNOS expression attenuation in the 7 day old and 21 day old, but not in the adult. AT2 receptor inhibition attenuated nNOS expression the greatest in the newborn and 21 day old afferent arterioles reducing nNOS expression by 52% and 65%, respectively. However, it was observed that AT2 receptor inhibition did not significantly attenuate nNOS expression in the afferent arterioles of neither the 7 day old nor the adult. Since basal levels of nNOS in the afferent arterioles of the adult are very low and not believed to play a critical role in adult renal hemodynamics, the lack of nNOS regulation via the AT1 and AT2 receptors should be expected. However, it was surprising to see inhibition of the AT2 receptor in the afferent arterioles of the 7 day old kidney did not attenuate nNOS expression. While results from the third series of experiments in this dissertation indicated AT2 receptor expression was low in the 7 day old preglomerular microvessels, the AT1 receptor was significantly increased in expression at 7 days. This may suggest the AT1 receptor is unregulated in the 7 day preglomerular microvessels to regulate nNOS expression.
during a period when AT2 receptor expression is low. Based on the studies here, nNOS expression is not regulated by AII via the AT2 receptor in afferent arterioles of the 7 day old kidney, a period when AT2 receptor expression is at its lowest in the neonate kidney. This suggests a possible cross signaling between the AT1 and AT2 receptor in the immature kidney vasculature.

Similar to what was seen regarding eNOS expression, at ages when the expression of the AT1 and AT2 receptor was low, inhibition of either receptor still resulted in significant attenuation of nNOS expression. The results in this study indicate nNOS expression is regulated by AII via both the AT1 and AT2 receptors, but the AT1 receptor, even though decreased in expression in the immature kidney, has greater influence on nNOS expression than does the AT2 receptor in the afferent arterioles of the developing kidney. It was previously believed the AT1 receptor was responsible for the vasoconstrictor response to AII while the AT2 elicited the AII vasodilation response. The results from this study indicate that both receptors regulate the NOS vasodilation response to AII stimulation.

The results of the NOS enzymatic catalytic activity assay indicated inhibition of either receptor, both AT1 and AT2, lead to attenuation of NO production in both the newborn and the adult. However, inhibition of the AT1 receptor resulted in greater reduction of NO production in the adult while inhibition of the AT2 receptor resulted in greater attenuation of NO production in the newborn. It appears AII regulates NO formation by either receptor in the newborn afferent arteriole, but more so through the AT2 receptor. Taken collectively with the expressional data, AII regulates eNOS and nNOS mRNA expression and NOS enzymatic activity in the afferent arterioles of the
immature kidney through both AT1 and AT2 receptors, but expression is more influenced by the AT1 receptor, while NOS enzymatic activity is more influenced by the AT2 receptor. The results in this forth series of experiments support the hypothesis that AII regulates NOS expression and function via the AT1 and AT2 receptors.

Overall, the studies in this dissertation were able to identify expressional and functional developmental patterns for both nNOS and eNOS in the preglomerular resistance vessels and glomeruli of the neonate. The expression of eNOS was low in both preglomerular vessels and glomeruli in the neonate and progressively increased with maturation suggesting eNOS may contribute to the renal hemodynamics of the neonate, but is not the critical NOS isoform in the neonate’s vasculature. The expression of nNOS, while absent in the glomerulus, was high in the neonate’s preglomerular resistance vessels and decreased with maturation suggesting the role of nNOS is enhanced in neonate renal hemodynamics. Functional studies showed NO production was elevated in all preglomerular vessels of the neonate, with decreased NO production occurring with maturation. In the neonate’s preglomerular resistance vasculature, when eNOS expression is low and nNOS expression is high, NO production is at its greatest. Studies observing AII regulation of NOS were able to provide evidence that both eNOS and nNOS mRNA expression and NOS enzymatic catalytic activity are regulated by AII through the AT1 and AT2 receptors, particularly in the afferent arterioles. Whole animal studies using the NOS unspecific inhibitor L-NAME and the nNOS specific inhibitor L-SMTC indicated nNOS is the major NOS isoform that is crucial to neonate renal hemodynamics. Thus, both whole animal studies utilizing NOS inhibitors and novel microdissection techniques aimed at analyzing
expression and function of NOS isoforms suggest nNOS is the crucial NOS isoform in the immature preglomerular resistance vessels.

Overall conclusions of this dissertation include: nNOS is the major isoform regulating renal hemodynamics in the immature, but not the mature, kidney. eNOS expression is developmentally regulated in glomeruli with differences found between intracortical location of glomeruli. NOS enzymatic activity in preglomerular resistance vessels is greater in the newborn than in the adult. This increase in enzymatic activity may be due to the upregulation of nNOS expression that was observed in the preglomerular resistance vessels. While AII regulates eNOS expression and NOS enzymatic activity, via the AT1 and AT2 receptors, in preglomerular resistance vessels of both newborn and adults, nNOS expression is regulated by AII only in the newborn.

The significance of the findings in this dissertation ultimately may impact the care given to neonates with compromised health conditions. Blockade of AII through AT1 receptor inhibition in the immature kidney significantly downregulates NOS expression. This is an important finding for possibly uncovering the role of NO in two types of neonatal ARF, ACE inhibitor-related ARF and vasomotor ARF. Angiotensin Converting Enzyme, ACE, inhibition has been well described to produce ARF in the neonate, in the absence of other mitigating factors. Administration of the Angiotensin Converting Enzyme ACE, inhibitor, either prenatally or in the mother or in the neonate (139) is associated with oligo-anuric acute renal failure. Until now, the mechanism causing the ARF from the perinatal and neonatal use of ACE inhibitors was not well understood. The observations that AT1 receptor inhibition downregulates NOS in the postnatal developing kidney provides a possible pathophysiologic basis for this type of
neonatal ARF. Perhaps with ACE inhibition, the neonatal kidney loses the upregulatory action of AII on NOS isoforms resulting in a decrease in NOS expression and function. Neonatal ACE downregulation of NOS occurs at a time when NO is a critical modulator of renal hemodynamics, thus the immature kidney loses the protective NO vasodilation (139). With ACE administration, even though AII vasoconstriction is alleviated, other highly activated vasoconstrictors operant in the neonatal kidney, such as endothelin, are functioning unopposed by NO (14, 120). These findings may not only lead to further caution in the use of ACE or AT1 receptor antagonists in the newborn, but also direct attention to NOS as a possible beneficial factor in preventing or treating this type ARF in the newborn.

The newborn kidney is vulnerable to vasomotor ARF from adverse pathologies that further decrease RBF, such as the hypotension and hypoxia of perinatal asphyxia, or complications of prematurity. These conditions induce an intense renal vasoconstriction, considered to be mediated by AII, resulting in vasomotor ARF (6, 139). NO vasodilation is vitally protective in this type of ARF. The protective role of NO, and its relationship with regulatory factors in vasomotor ARF have not been examined. Currently, therapeutic options are limited in the care of the neonate with vasomotor acute renal failure from any perinatal cause (139). Thus, studying the regulatory relationship of AII and NO may lead to the prevention or therapy of neonatal vasomotor ARF.
REFERENCES


APPENDIX A

To whom it concerns,

I am a graduate student at Eastern Virginia Medical School / Old Dominion University. I am on the verge of graduating and have been preparing my dissertation. I conducted my graduate research in the lab of Dr. Michael Solhaug, at Eastern Virginia Medical School. In our lab, we study renal hemodynamics in the newborn. In 2000 and 2001, two papers from our lab were published in the American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. In my dissertation, I hope to include multiple figures from those two papers with your permission. The 2000 paper is titled "Ontogeny of neuronal nitric oxide synthase, NOS I, in the developing porcine kidney" (Vol. 278, Issue 6, R1453-R1459, June 2000). The figures I would like to include in my dissertation are figures 2, 3 and 4. The second paper, from 2001, was titled "Expression of endothelial nitric oxide synthase in the postnatal developing kidney" (Vol. 280, Issue 5, R1269-R1275, May 2001). The figures I would like to include in my dissertation from the second paper are figures 1-4. My mentor, Dr. Michael Solhaug, is the primary author on both of the papers. This letter is to serve as a formal request for permission to include in my dissertation the figures mentioned that have been previously published in the American Journal of Physiology - Regulatory, Integrative and Comparative Physiology.

Thank you,

Brian Ratliff
3/20/06

The American Physiological Society
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<th>Institution and Location</th>
<th>Degree</th>
<th>Year(s)</th>
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<tr>
<td>Chowan College, Murfreesboro, NC</td>
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Selected Abstracts