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# **Role of Nitric Oxide (NO) and Regulation of NO Synthesis in the Kidney**

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Thesis submitted to the Department of Cellular and Molecular Medicine, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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1999



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## DEDICATION

*To my loving parents Krystyna and Marek Roczniak*

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I am deeply indebted to my supervisor, Dr Kevin Burns, for his guidance and support for the past six years. He has truly provided an excellent environment for scientific development and independent thought. His advice and friendship are greatly appreciated.

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## ABSTRACT

The general objective in undertaking this study was to determine the role of nitric oxide (NO) and neuronal nitric oxide synthase (nNOS) in the regulation of Na<sup>+</sup> excretion by the kidney. NO regulates renal hemodynamics and induces natriuresis. The induction of Na<sup>+</sup> excretion may be caused by either vasodilatory action of NO, and/or its direct effect on the renal tubules. Our study was the first to show that in the proximal tubule NO donors inhibit the NHE3-mediated Na<sup>+</sup> uptake in a cGMP-dependent manner. This is an important observation since the proximal tubule is a major site of salt and water reabsorption in the kidney, and the apical NHE3 exchanger is the most important transporter of Na<sup>+</sup> in the proximal tubule. Possible implications of these findings are for the regulation of the extracellular volume and development of hypertension.

Nitric oxide is produced by a family of enzymes called nitric oxide synthases. The nNOS has been implicated in the long-term regulation of blood pressure. We conducted the next study to determine the effect of dietary salt on the expression of this important NOS isoform. We determined that nNOS is localized in the macula densa of the renal cortex and in the inner medullary collecting ducts (IMCD), by immunohistochemistry. Our data also shows that high dietary NaCl differentially regulates nNOS mRNA and protein expression in the IMCD and renal cortex. Whereas high dietary NaCl inhibits nNOS mRNA and protein expression in the cortex, it increases nNOS protein expression without affecting mRNA levels in the IMCD. Our study is the first to identify cell-specific regulation of nNOS in the kidney and suggests that in the cortex and inner medulla nNOS function might be different.

Chronic renal failure (CRF) is characterized by low NO production, but the mechanism for decreased NO production is unknown. This could have important implications, since impaired NO production might contribute to increased blood pressure. Accordingly, we determined the effect of 5/6 nephrectomy in rats (5/6 Nx, a model of chronic renal failure) on intrarenal expression of nNOS. Our study shows that in the cortex and inner medulla of 5/6 Nx rats, nNOS mRNA and protein expression are significantly reduced, suggesting that the low NO production in CRF might be in part due to decreased nNOS expression. In addition, we tested whether angiotensin II (Ang II) might be responsible for downregulating nNOS expression in CRF. Although Ang II was not responsible for downregulating nNOS expression, we uncovered a previously unknown interaction between the renin-angiotensin II and nitric

oxide systems. We found that in normal kidneys Ang II acts through AT<sub>1</sub> receptors to tonically upregulate nNOS expression. This is an important observation since both of these systems are important regulators of renal hemodynamics and Na<sup>+</sup> excretion.

Recently, a novel 89 amino acid protein (PIN- protein inhibitor of nNOS) was isolated from rat brain and shown to inhibit nNOS activity. By reverse transcription polymerase chain reaction, PIN mRNA was detected in the kidney cortex and inner medulla. Immunohistochemistry revealed staining for PIN in endothelial cells of glomeruli and vasa rectae. PIN was also localized to the apical membranes of inner medullary collecting duct (IMCD) cells. Two weeks after 5/6 Nx, inner medullary PIN expression was significantly upregulated as determined by Western blotting. These data show that PIN, a specific inhibitor of nNOS activity, is expressed in the IMCD, a site of high nNOS expression in the kidney. PIN expression is upregulated in the inner medulla of 5/6 Nx rats. Inhibition of nNOS activity by PIN may have important implications for the regulation of NO synthesis in the IMCD of normal and remnant kidneys.

In conclusion, we have demonstrated that NO regulates Na<sup>+</sup> uptake by directly inhibiting the NHE3 in the PT, suggesting that this could be one of the mechanisms responsible for the induction of natriuresis by NO. Our studies further demonstrated that nNOS expression in the cortex and IMCD is differentially regulated by high dietary salt, suggesting regulation from different, cell-specific promoters. In 5/6 Nx rats, we demonstrated that nNOS mRNA and protein are significantly downregulated and PIN, a specific nNOS inhibitor is upregulated, which could contribute to the overall low NO production in the kidneys of rats with CRF. Treatment with losartan had no effect on the expression of nNOS in the inner medulla of 5/6 Nx rats but reversed, in part, the inhibitory effect of 5/6 Nx on nNOS in the cortex. Furthermore, Ang II may tonically upregulate nNOS expression in the cortex and the inner medulla.

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## LIST of ABBREVIATIONS

Ang II; angiotensin II  
ANP; atrial natriuretic peptide  
AT1; angiotensin II type I receptor  
AT2; angiotensin II type II receptor  
AVP; arginine vasopressin  
BUN; blood urea nitrogen  
cGK; cGMP-dependent kinase  
CaM; calmodulin  
cAMP; cyclic adenosine monophosphate  
CCD; cortical collecting duct  
CD; collecting duct  
cGKII; cGMP dependent protein kinase type II  
cGMP; cyclic guanosine monophosphate  
CRF; chronic renal failure  
DCT; distal convoluted tubule  
ecNOS; endothelial nitric oxide synthase  
EDRF; endothelium derived relaxing factor  
EnaC; epithelial sodium channel  
GFR; glomerular filtration rate  
IMCD; inner medullary collecting duct  
iNOS; inducible nitric oxide synthase  
JGA; juxtaglomerular apparatus  
Kf; filtration coefficient  
L-NAME; N<sup>G</sup>-nitro-L-arginine  
MAP; mean arterial pressure  
mRNA; messenger RNA  
mTAL; medullary thick ascending limb  
NHE3; Na<sup>+</sup>/H<sup>+</sup> exchanger 3  
nNOS; neuronal nitric oxide synthase  
NO; nitric oxide  
NOS; nitric oxide synthase  
5/6 Nx; 5/6 nephrectomy  
OMCD; outer medullary collecting duct  
P<sub>B</sub>; Bowman's space pressure  
PDE; phosphodiesterase  
P<sub>GC</sub>; glomerular capillary pressure  
PIN, protein inhibitor of nNOS  
PT; proximal tubule  
Π<sub>B</sub>; Bowman's space oncotic pressure  
Π<sub>GC</sub>; glomerular capillary oncotic pressure  
RAS; renin-angiotensin II system  
RBF; renal blood flow  
RMR; renal mass reduction  
RPF; renal plasma flow  
sGC; soluble guanylate cyclase  
SNAP; S-nitroso-N-acetylpenicillamine  
SNP; sodium nitroprusside  
TAL; thick ascending limb  
TGF; tubuloglomerular feedback

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## THESIS FORMAT

This thesis is submitted as compilation of published or submitted journal articles (Chapters 2 to 5). Chapter 1 contains a general overview of relevant literature, whereas chapter 6 contains a general discussion. All references are found in chapter 7.

## CHAPTER 1: LITERATURE REVIEW

### 1.1 Nitric Oxide and its Synthesis

The 1998 Nobel Prize in Physiology and Medicine was awarded to F. Murad, L. J. Ignarro, and R. F. Furchgott who elucidated the role of nitric oxide (NO) in human biology. In the past decade close to 30,000 papers have been written on NO, illustrating the interest that this molecule sparked in the research community. NO is one of the smallest biological mediators identified to date and is generated by a family of nitric oxide synthases in a reaction unprecedented in its complexity. Intensive investigation over the decade demonstrated that NO is a neurotransmitter and a mediator of memory formation in the central nervous system, an antibacterial and antitumor factor, a regulator of blood pressure, of skeletal muscle contractility and of renal function. Yet, uncontrolled NO production contributes to tissue damage in arthritis, diabetes, glomerulonephritis, septic shock, and stroke.

#### 1.1.1 Nitric Oxide

It has been recognized since 1916 that tissues are capable of producing nitrates (Kelm, 1999). This fact was ignored until Green et al. showed that nitrate production in mammals is enhanced by infection or treatment with endotoxin (Green et al., 1981; Green et al., 1981). Meanwhile, Furchgott et al. identified a substance released from endothelial cells that mediates relaxation of blood vessels (Furchgott and Zawadzki, 1980) and called it the endothelium derived relaxing factor (EDRF). In 1987, EDRF was shown to be NO by two separate laboratories (Ignarro et al., 1987; Palmer et al., 1987). The nitrates, as measured by Green et al., are the degradation products of NO produced in mammalian cells (Palmer et al., 1988).

It is now well established that NO is produced in mammalian cells at concentrations ranging from 10 nM to 1  $\mu$ M (Kelm, 1999). The diffusion of NO from one cell to another is facilitated by its hydrophobic nature (Subczynski et al., 1996). The rate of diffusion of NO is in fact greater than that of O<sub>2</sub>

when measured in aqueous solution at 37°C ( $3300 \mu\text{m}^2 \text{s}^{-1}$ ) (Kelm, 1999). However, NO possesses an unpaired electron and is a fairly reactive free radical. Indeed, NO reacts rapidly with other radicals such as oxygen and superoxide, as well as with ferro-hemoproteins, and thiol-containing molecules (Kelm, 1999). Due to its reactivity NO half-life varies greatly (from seconds to minutes) depending on the system in which it is measured (Kelm, 1999). Hence, the environment in which it is synthesized, namely the concentration of its bioreactants and the redox potential of the tissue, determines NO half-life and limits its diffusion distance.

### 1.1.2 NO Synthesis

**Nitric oxide synthase (NOS).** A family of enzymes termed nitric oxide synthases catalyze NO production in cells. The family comprises three isoforms termed neuronal NOS (nNOS/NOSI) (Bredt et al., 1991), endothelial NOS (ecNOS/NOSIII) (Marsden et al., 1992) and inducible NOS (iNOS/NOSII) (Lowenstein et al., 1992). NOS monomers are bidomain proteins, formed by the C-terminal reductase domain and the N-terminal oxygenase domain, separated by a calmodulin-binding motif (Bredt et al., 1991; Stuehr, 1999). The bidomain structure of NOS is further subdivided into distinct functional modules comprised of the binding sites for NADPH, FAD, FMN, heme, and tetrahydrobiopterin ( $\text{H}_4\text{B}$ ) (Stuehr, 1999). In their active state, NOS isoforms exist in a homodimeric form (Masters et al., 1996) that is mediated by the interaction of the oxygenase domains (Chen et al., 1996). NOS dimers use L-arginine and  $\text{O}_2$  as substrates to generate NO and L-citrulline in equimolar proportion (Stuehr, 1999).

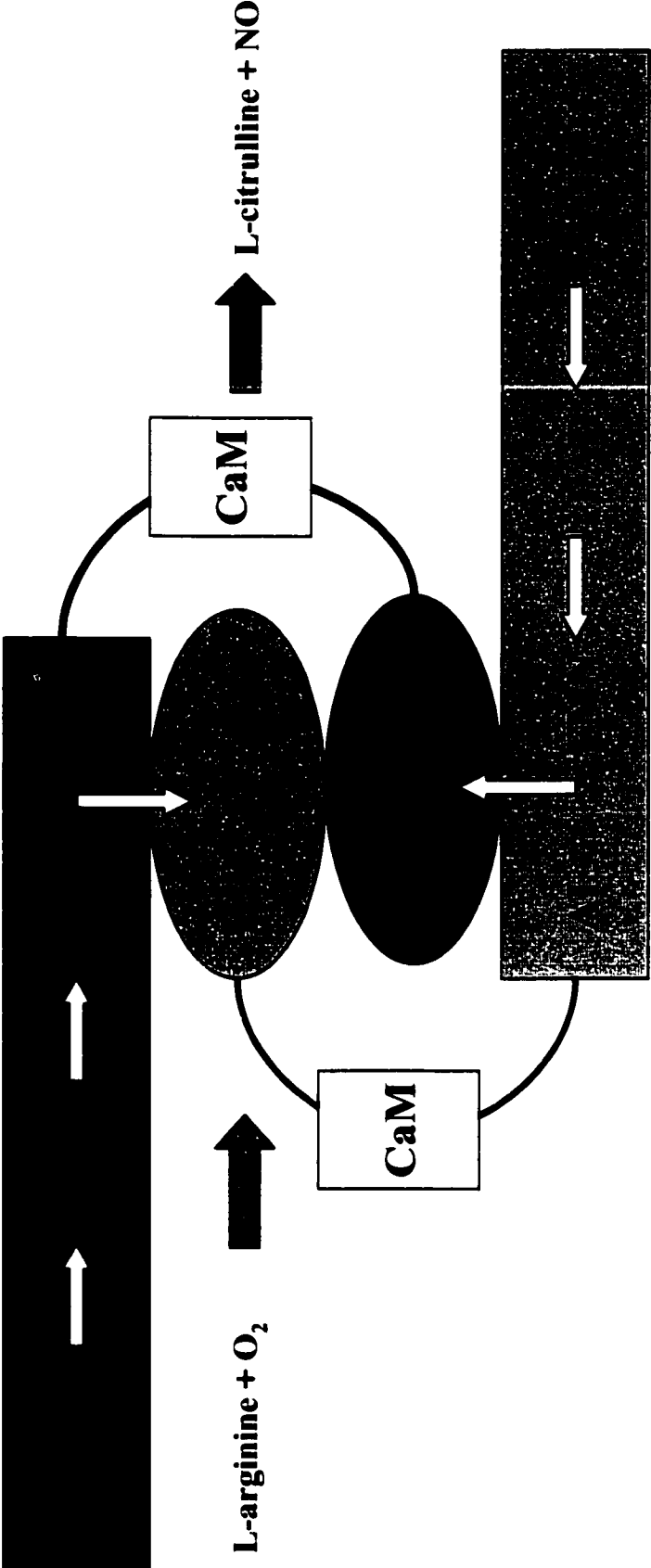
**NOS cofactors.** The mechanism of NO synthesis is depicted in **Figure 1.1**. The binding sites for NADPH, FAD, and FMN are found in the reductase domain of NOS (Masters et al., 1996). The main function of these modules is the transfer of electrons from NADPH to both FAD and FMN (Masters et al., 1996; Stuehr, 1999). The flavins then release electrons to the heme-iron module localized in the oxygenase domain of the adjacent subunit of NOS where NO synthesis takes place (Stuehr, 1999). Interestingly, electron flow between reductase and oxygenase domains on the same subunit does not occur (Siddhanta et al., 1998).

In addition to the heme binding site, the active site for NO formation also contains binding sites for  $\text{H}_4\text{B}$ , and L-arginine (Stuehr, 1999). Several studies have demonstrated that the heme is present in

purified NOS and is required to obtain maximal catalytic activity (Stuehr and Ikeda-Saito, 1992), possibly by facilitating dimerization of NOS monomers (Baek et al., 1993). Moreover, studies indicate that the heme is the site of oxygen activation for oxidation of L-arginine (Frey et al., 1994; Wolff et al., 1993). The role of H<sub>4</sub>B is not as well understood, but it may modulate oxygen-heme reactivity (Abu-Soud et al., 1997).

The role of calmodulin (CaM), which joins the reductase and oxygenase domains, is particularly intriguing. CaM, a protein that contains two globular binding sites for Ca<sup>2+</sup>, is essential for electron flux between the reductase and oxygenase domains of ecNOS and nNOS (Abu-Soud et al., 1994). The mechanism by which CaM increases NO synthesis is still unknown but studies indicate that CaM alters the conformation of the reductase domain (Gachhui et al., 1996), and increases the rate of electron transfer into the flavins in nNOS (Gachhui et al., 1998). A putative autoinhibitory domain has been identified within the FMN domain and next to the CaM binding site of both nNOS and ecNOS (Salerno et al., 1997). This insert (approximately 50 amino acids) inhibits CaM binding to ecNOS and nNOS and also contributes to the low overall activity of ecNOS (Nishida and Ortiz de Montellano, 1999). In both ecNOS and nNOS, deletion of the insert significantly decreases Ca<sup>2+</sup> concentrations required to activate the enzymes (Nishida and Ortiz de Montellano, 1999). The inducible NOS lacks the autoinhibitory insert found in ecNOS and nNOS and is tightly associated with CaM (Cho et al., 1992). Perhaps for this reason, iNOS is relatively insensitive to intracellular Ca<sup>2+</sup> concentrations (Ruan et al., 1996; Venema et al., 1996).

**Figure 1.1: Figure 1.1: Domain arrangement of a NOS dimer.** The reductase domain of NOS isoforms contains binding sites for NADPH, FAD, and FMN. These cofactors are responsible for the electron transfer (indicated by arrows) to the oxygenase domain where NO synthesis takes place. The electron transfer between the reductase and oxygenase domains is dependent on CaM binding. Binding of CaM is hindered by an autoinhibitory domain (AI) present in both ecNOS and nNOS but not iNOS. The oxygenase domain contains binding sites for heme (Fe), L-arginine (Arg), and tetrahydrobiopterin (H<sub>4</sub>B). In their active form the NOS isoforms form homodimers due to the interaction of the oxygenase domains. This figure was adapted from Stuehr et al. (Stuehr, 1999).



### 1.1.3 Nitric Oxide Synthases

The structural organization of NOS isoforms is depicted in **Figure 1.2**. The human nNOS (160 kDa), iNOS (130 kDa) and eNOS (135 kDa) proteins are encoded by separate genes which are located on human chromosomes 12, 17, and 7, respectively (Stuehr, 1999). Analysis of the genomic organization and exon/intron structure, including exon sizes and the locations of the intronic splice junctions, revealed a high degree of similarity among the three genes (Hall et al., 1994). The high degree of conservation among the NOS isoforms suggest recent duplication and chromosome transposition events (Hall et al., 1994). The primary amino acid sequences of NOS proteins is also similar and they share approximately 50% amino acid sequence identity (Lamas et al., 1992). Within each isoform group, there is a high degree of amino acid identity across species (80-94%) (Lamas et al., 1992). The only related gene in the human genome is the cytochrome P-450 reductase. The similarities between NOS and P-450 are particularly evident in the exons implicated in binding NADPH (Bredt et al., 1991). The three isoforms of NOS mostly differ with respect to their localization, regulation and function.

The cDNA for nNOS was the first of the three isoforms to be cloned (Bredt et al., 1991; Hall et al., 1994). Although nNOS was originally cloned from the brain, other tissues including testes (Bredt et al., 1991), skeletal muscle (Brenman et al., 1995), and kidney epithelial cells (Wilcox et al., 1992) also express nNOS. In neurons (Brenman et al., 1996) and skeletal muscle (Brenman et al., 1995) nNOS is membrane-associated but it can also be localized in the cytosol, as is the case in kidney epithelial cells (Wilcox et al., 1992). Neuronal NOS knockout mice (nNOS<sup>-/-</sup>) have dilated stomachs with a constricted pyloric sphincter (Huang et al., 1993), are resistant to brain damage caused by vascular strokes (Huang et al., 1994), are highly aggressive and display excessive sexual behaviour (Nelson et al., 1995). These studies suggest that nNOS regulates gastrointestinal function and sexual behaviour and that excessive NO production by nNOS contributes to neuronal damage in stroke.

**Figure 1.2: Alignment of the deduced amino acid sequences of the three isoforms of NOS.** Depicted are the consensus binding sites for heme, L-arginine, calmodulin (CaM), FMN, FAD, and NADPH. The N-terminal of nNOS contains an additional 200 amino acid leader sequence which contains the PDZ domain and the binding site for the protein inhibitor of nNOS (PIN). The N-terminal of ecNOS, on the other hand is myristoylated (myr) and palmitoylated (pal). Note that the structural organization of iNOS and ecNOS are similar except that they lack the leader sequence expressed in nNOS and that iNOS lacks the autoinhibitory loop (AI) expressed in both ecNOS and nNOS. The numbers below each NOS sequence represent exon numbers. This figure was adapted from Gross et al. (Gross, 1997).



nNOS

PDZ PIN H BH4 Arg AI FMN FAD NADPH



2-3 4-5 6 7-9 10-12 13-14 15 16-17 18-20 21-23 24-29

ecNOS

Pal < />  
Myr < />



1 2-3 4 5-7 8-10 11-12 13 14-15 16-18 19-21 22-26

iNOS



2-3 4-5 6 7-9 10-11 12-13 14-18 19-21 22-26

The ecNOS was originally purified and cloned from endothelial cells and is the isoform responsible for producing the EDRF as described by Furchgott et al (Furchgott and Zawadzki, 1980). Cloning of the human and bovine ecNOS revealed that it lacks a TATA box (Janssens et al., 1992; Lamas et al., 1992; Marsden et al., 1992; Sessa et al., 1992; Venema et al., 1994) a feature commonly found in genes that are under tight transcriptional control and not cytokine activated. Expression of ecNOS is essentially restricted to vascular endothelial cells (Kone, 1999). In the endothelial cells ecNOS is targeted to the cellular areas called the caveolae, where it associates with the plasma membrane (Feron et al., 1998). Endothelial NOS knockout mice (ecNOS<sup>-/-</sup>) demonstrate a 35% increase in mean blood pressure as compared to wild-type animals (Huang et al., 1995). In addition, the aortic rings display no relaxation in response to acetylcholine and are unaffected by NOS inhibitors (Huang et al., 1995). The pronounced hypertension of the ecNOS<sup>-/-</sup> mice establishes ecNOS as an important determinant of blood pressure, producing a major vasodilator.

The inducible NOS was originally cloned from cytokine-stimulated macrophages (Geller et al., 1993; Lowenstein et al., 1992; Lyons et al., 1992; Xie et al., 1992). It sharply differs from nNOS and ecNOS in that its expression is increased by cytokines and lipopolysaccharide (LPS) and repressed by glucocorticoids (Kone, 1999). The promoter of the iNOS gene is divided into two functional regions. Region I is a core promoter, whereas Region II is an LPS- and cytokine- responsive enhancer (Lowenstein et al., 1992). In most cells iNOS is synthesized in response to inflammatory or proinflammatory mediators (Kroncke et al., 1995). In select tissues however, including kidney epithelial cells, iNOS mRNA is constitutively expressed (Mohaupt et al., 1994). In most cells iNOS protein is localized in the cytosol (Kone, 1999). The iNOS knockout mice (iNOS<sup>-/-</sup>) have markedly reduced defences against microorganisms such as *Listeria* (MacMicking et al., 1995; Wei et al., 1995), and against proliferation of lymphoma tumour cells (MacMicking et al., 1995). On the other hand, iNOS<sup>-/-</sup> mice are resistant to carrageenan inflammation (Wei et al., 1995) and hypotension elicited by endotoxin (MacMicking et al., 1995; Wei et al., 1995). In the kidney, iNOS is involved in glomerular inflammatory diseases such as glomerulonephritis (Kone et al., 1997). These studies suggest that iNOS may play a role in host defence or in modulating the immune response. The role of the constitutively expressed iNOS in kidney cells is unknown.

### 1.1.4 Neuronal NOS

**The gene.** The human nNOS gene occupies more than 200 kb of DNA on chromosome 12 and consists of 29 exons and 28 introns (Hall et al., 1994). The full-length open reading frame is 4302 base pairs, encoding a protein of 1434 amino acids (Bredt et al., 1991; Hall et al., 1994). The 5'-flanking region of the nNOS gene contains multiple cis-acting elements such as AP-2, TEF-1/MCBF, CREB/ATF/c-fos, NRF-1, Ets, NF-1, and NF- $\kappa$ B binding motifs (Hall et al., 1994). However, it is still unclear which of these elements in the promoter region contribute to the transcriptional regulation of nNOS expression. Dinucleotide (dC-dA)<sub>n</sub> repeats are found in the 5'-flanking region of exon 1, the 5' portion of intron 2, and within exon 29 (Hall et al., 1994). Variations in the dinucleotide repeats in the 5' untranslated region (5'UTR) may cause differences in basal promoter activity due to spacing effects or double-helix secondary structure, while the presence of exonic dinucleotide repeats suggests the existence of allelic mRNA diversity (Hall et al., 1994). Indeed, genotypic analysis of a number of normal individuals indicates the existence of multiple alleles that vary in size dependent on dinucleotide repeats (Hall et al., 1994). The role of dinucleotide repeats in the human genome remains to be determined. Of interest however, is the observation that some neurodegenerative disorders are caused by the expansion of unstable nucleotide repeats (Carango et al., 1993).

**Molecular diversity of nNOS.** The nNOS is the most structurally diverse human gene described to date. The nNOS gene produces multiple mRNA transcripts via alternative promoter usage, alternative splicing, cassette insertions/deletions and varied sites for 3'-untranslated region (3'UTR) cleavage and polyadenylation (Hall et al., 1994). At least nine different types of exon 1 have been isolated and are spliced to a common exon 2 (Hall et al., 1994). In addition, a kidney specific exon 1 has been identified by Oberbaumer et al (Oberbaumer et al., 1998). Each of the alternative forms of exon 1 is flanked by its unique 5'-upstream sequence, suggesting that unique promoters drive nNOS expression. To date, two separate promoters that drive transcription were identified in nNOS transfected cells (Xie et al., 1995). Since the translational start site is found in exon 2, all of these transcripts should give rise to the same protein (Hall et al., 1994). The advantage of such complexity is not obvious, but it may facilitate tissue-specific and/or developmental regulation of nNOS expression. Indeed, nNOS 5'UTRs contain cis RNA

elements that modulate translational efficiency in responses to changes in cellular phenotype (Wang et al., 1999). The organization of nNOS 5'UTR is depicted in **Figure 1.3A**.

In addition to the differences in the 5'UTR of nNOS mRNA, various cassette deletions in nNOS transcripts have been identified. These include splicing-out of exons 9/10 and 10 (Fujisawa et al., 1994; Hall et al., 1994; Ogura et al., 1993). Spliced variants of exons 9 and 10 results in an mRNA that is 315 base pairs shorter, and results in a protein lacking 105 amino acids located at the N-terminal of the CaM binding domain (Hall et al., 1994). Variants lacking exon 10 have also been described, and have been identified in the kidney, but are expected to yield an inactive protein because a premature stop codon is introduced (Hall et al., 1994).

Tissue-specific alternatively spliced isoforms of nNOS have been identified in skeletal muscle (Silvagno et al., 1996), testes (Wang et al., 1997), and brain (Brenman et al., 1996). In skeletal muscle, the novel nNOS protein, termed nNOS  $\mu$  contains an additional 102 base pairs between exons 16 and 17 (Silvagno et al., 1996). This additional sequence encodes a 34 amino acid sequence which may contain putative phosphorylation sites for muscle-specific regulation (Brenman et al., 1997). Furthermore, two alternatively spliced isoforms, nNOS  $\beta$  and nNOS  $\gamma$ , lacking exon 2 have been identified in the brain (Brenman et al., 1996). The nNOS  $\beta$  transcript has also been detected in the kidney (Oberbaumer et al., 1998). The role of alternatively spliced isoforms of nNOS is unknown but they may be important for specific regulation during development, as is the case in skeletal muscle (Silvagno et al., 1996). These isoforms may also be important for tissue-specific regulation and intracellular localization. Tissue-specific isoforms of nNOS are depicted in **Figure 1.3B**.

In summary, nNOS expression is regulated by intricate mechanisms which consist of the use of tissue-specific promoters, regulation of translational efficiency by the various 5'UTRs, and the alternative splicing of the mRNA in a tissue-specific manner. This complexity is probably necessary to allow tissue-specific regulation of nNOS expression and activity and may explain the multitude of functions performed by nNOS in different tissues. It should be noted that kidney-specific isoforms of nNOS have not been identified.

**Figure 1.3: Molecular diversity of nNOS.** (A) Depicts the alternative promoter usage for human nNOS. Each exon 1 is regulated by a different promoter/enhancer. The alternative use of exon 1 is tissue-specific. Each exon 1 is spliced into a common exon 2, thus one gene produces many mRNA molecules but only one protein product. Translational efficiency of nNOS is modulated by 5'UTR cis RNA elements in a tissue-specific manner (Wang et al., 1999). (B) Depicts nNOS isoforms which are alternatively spliced in a tissue-specific manner to generate different proteins. Alternative splicing of nNOS mRNA may be important for tissue-specific regulation of nNOS activity (Brenman et al., 1997).



**The PDZ Domain of nNOS.** Exon 2 of nNOS encodes an N-terminal extension of nNOS containing a PDZ domain (Brenman et al., 1996). The PDZ domain is named after three proteins: the postsynaptic density protein PSD95/SAP90, the *Drosophila* disc-large tumor suppressor, and the tight junction protein ZO-1, all of which bear the signature consensus sequence GLGF (Fanning and Anderson, 1999). The PDZ/GLGF motif participates in protein-protein interactions (Harrison, 1996) and targets nNOS to membrane structures (Brenman et al., 1995). Studies indicate that PDZ domains may be important for mediating interactions required for signal transduction at the membrane (Fanning and Anderson, 1999). The alternatively spliced isoforms of nNOS, nNOS  $\beta$  and  $\gamma$ , lack exon 2 (**Figure 1.3B**) and therefore do not express the PDZ domain (Brenman et al., 1996). The intracellular localization and signaling of these proteins might therefore differ from nNOS (Brenman et al., 1996). Targeting of nNOS protein to various intracellular proteins is an important mechanism for regulating nNOS activity and localizing NO generation to particular areas of the cell. This particular aspect of nNOS biology will be discussed in the next section.

### 1.1.5 Regulation of nNOS Activity

**Regulation by protein-protein interactions.** Due to the nature of NO reactivity and to the deleterious effects of excessive NO generation, nNOS protein activity is very tightly regulated. Protein-protein interactions are the principal mechanism of nNOS activity regulation. For instance, in the brain, the nNOS PDZ domain associates with the postsynaptic density (PSD) proteins PSD-93 and PSD-95 (Brenman et al., 1996). This interaction physically links nNOS to N-methyl-D-aspartate (NMDA) receptors that are also associated with PSD95 (Christopherson and Brecht, 1997).  $\text{Ca}^{2+}$  influx through the NMDA receptors activates nNOS (Christopherson and Brecht, 1997). Similarly, the PDZ domain of nNOS associates with the dystrophin complex in skeletal muscle (Brenman et al., 1995) and couples nNOS activity with muscle membrane depolarization and the activity of acetylcholine receptors (Christopherson and Brecht, 1997). There are two consequences of nNOS targeting in neurones and skeletal muscle, one is that nNOS activity can be rapidly regulated and the second is that NO generation occurs in localized areas of the cell.

Neuronal NOS also interacts with caveolins localized in caveolae. Caveolae are small invaginations in the plasma membrane characterized by a distinctive lipid composition (enriched in

cholesterol and glycosphingolipids) and by the presence of the structural "scaffold" protein called caveolin (Anderson, 1993). In many tissues, caveolae may serve as sites for the sequestration of signaling molecules such as receptors, G proteins and protein kinases (Anderson, 1993; Michel and Feron, 1997). Neuronal NOS interacts with caveolin-1 and caveolin-3 *in vitro* (Venema et al., 1997). Furthermore, synthetic peptides corresponding to regions of the two caveolins potently inhibit the catalytic activity of purified, recombinant nNOS (Venema et al., 1997). The role of the association of nNOS and caveolins *in vivo* is not known, but these studies suggest at least two possible ways by which nNOS activity may be regulated. One possibility is a direct inhibition of nNOS activity by caveolin binding, the second possibility is that the caveolins place nNOS in the proximity of cellular signalling pathways which may regulate nNOS activity by covalent modifications such as phosphorylation.

Finally, a recently isolated protein inhibitor of nNOS (PIN) may regulate nNOS activity (Jaffrey and Snyder, 1996). PIN is an 89 amino acid protein, initially cloned from the rat hippocampus. In HEK 293 cells, PIN inhibits  $\text{Ca}^{2+}$  ionophore-stimulated cGMP formation and nNOS activity (Jaffrey and Snyder, 1996). PIN binds to a 17 amino acid sequence of nNOS localized at the N-terminal of the oxygenase domain and destabilizes the dimeric structure of nNOS (Fan et al., 1998). Since the 17 amino acid sequence is absent from both ecNOS and iNOS, PIN may be a specific inhibitor of nNOS (Fan et al., 1998).

The protein associations of nNOS in the kidney are largely unknown. In embryonic 293 human kidney cells nNOS exists as a heterodimer with the heat shock protein hsp90 (Bender et al., 1999). This association may play a role in the regulation of nNOS activity and turnover (Bender et al., 1999), but its role *in vivo* has not been investigated. Another study colocalized nNOS and PSD-93 proteins in macula densa cells of the kidney (Tojo et al., 1999), but it is not known whether these proteins interact. Caveolin-3 has not been identified in the kidney but caveolin-1 is expressed in the principal cells of the cortical collecting duct (Breton et al., 1998), where it might associate with nNOS. In addition, the mRNA transcript for PIN has been identified in the kidney, but the precise site of its expression in the kidney and its role are unknown.

***Covalent modifications of nNOS.*** *In vitro* studies suggest that nNOS is regulated by the phosphorylation state of the enzyme. Phosphorylation of purified nNOS protein by PKA, PKC and  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase were shown to reduce its catalytic activity (Bredt et al., 1992;

Dawson et al., 1993; Dinerman et al., 1994). Calcineurin-mediated dephosphorylation of nNOS enhances its activity (Dawson et al., 1993).

**Regulation by L-arginine availability.** The circulating levels of L-arginine are approximately 100  $\mu\text{M}$ , which is substantially higher than the  $K_m$  of NOS for L-arginine (approximately 5  $\mu\text{M}$ ) (Venema et al., 1996). A cationic amino acid transport system termed “ $y^+$  transporter” facilitates uptake of L-arginine by cells (White, 1985). In addition, L-arginine can also be endogenously synthesized within cells, which includes conversion of L-citrulline to L-arginine by argininosuccinate synthetase (Hecker et al., 1990; Sessa et al., 1990). Changes in  $y^+$  transporter activity and intracellular L-arginine synthesis could potentially alter NO production.

**Pharmacological Inhibition of nNOS.** The activity of NOS enzymes is inhibited by L-arginine analogues such as  $\text{N}^G$ -nitro-L-arginine methyl ester (L-NAME) (Abu-Soud et al., 1994). The inhibition induced by L-arginine analogues is competitive and is readily reversed by excess L-arginine (Abu-Soud et al., 1994). Although these analogues inhibit iNOS, ecNOS and nNOS isoforms with different potencies they remain quite non-selective in their inhibition (Abu-Soud et al., 1994). 7-Nitroindazole (7-Ni) is currently used as an inhibitor of nNOS ( $\text{IC}_{50} = 710 \text{ nM}$ ) but it also inhibits ecNOS ( $\text{IC}_{50} = 800 \text{ nM}$ ). A highly specific inhibitor of nNOS has yet to be identified. Interestingly, an L-arginine analogue, termed asymmetric dimethyl arginine (ADMA) is synthesized endogenously and also non-selectively inhibits NOS activity (Vallance et al., 1992).

In summary, in addition to tight regulation of nNOS transcription and translation, nNOS protein is regulated by targeting to specific cellular areas where it may be coupled to  $\text{Ca}^{2+}$  channels, protein kinases or L-arginine transporters. In addition, proteins such as PIN and hsp90 bind to nNOS and inhibit its activity.

## 1.2 NO Signaling

### 1.2.1 The NO/cGMP Pathway

At physiological levels of NO, the primary target of NO is the soluble guanylate cyclase (sGC). The sGC is a heterodimeric protein composed of  $\alpha$  and  $\beta$  subunits, and is a member of the family of nucleotide cyclases that also includes adenylate cyclase and particulate guanylate cyclase (Denninger and Marletta, 1999). The particulate guanylate cyclase is membrane-associated, and is not activated by NO. The sGC on the other hand, localized in the cytosol of cells, is an important heme-containing target of NO (Denninger and Marletta, 1999). Activation of the sGC occurs upon binding of NO to ferrous heme and displacement of the histidine located on the opposite side of the heme ring. Displacement of the histidine leads to a conformational change of sGC (Traylor and Sharma, 1992) and results in the synthesis of cyclic guanosine 5'-monophosphate (cGMP). Mutation of the histidine to phenylalanine prevents activation of sGC by NO (Wedel et al., 1994).

There are three known targets for cGMP: the cGMP dependent kinase (cGK) (Lohmann et al., 1997), the cGMP-regulated phosphodiesterase (PDE) isoforms (Degerman et al., 1997), and the cGMP-gated ion channels which are found in a variety of tissues, most notably in the retina (Beavo, 1995; Zagotta and Siegelbaum, 1996). Mammalian cGKs exist in two major forms, the cytosolic cGKI, and the membrane associated cGKII (Lohmann et al., 1997). The targets for activated cGKs include ion channels and transporter proteins (Lohmann et al., 1997).

PDE isoforms decrease intracellular cAMP and cGMP levels by catalyzing the hydrolysis of the 3'-phosphodiester bond in cAMP and cGMP to yield AMP and GMP, respectively (Beavo, 1995). Three members of the PDE family namely PDE2, PDE5 and PDE6, are allosterically regulated by cGMP (Beavo, 1995) whereas the fourth member, PDE3 is inhibited by binding of cGMP to its substrate site (Degerman et al., 1997).

The sGC is not the only heme-containing target of NO. Other heme targets of NO include cyclooxygenase (Salvemini et al., 1993), cytochrome P-450 (Khatsenko et al., 1993), and the NOS heme prosthetic group, constituting a feedback inhibition loop (Kone, 1999). NO also acts as the inhibitor of

cytochrome c oxidase, thus inhibiting mitochondrial respiration (Lane and Gross, 1999) and binds to both hemoglobin and myoglobin (Jia et al., 1996). NO also reacts with enzymes containing nonheme iron. These enzymes include the iron-sulfur cluster enzymes such as aconitase (Drapier et al., 1993; Pantopoulos and Hentze, 1995; Weiss et al., 1993).

### **1.2.2 Thiol-containing Proteins**

Thiol-containing proteins are important signaling targets for NO (Stamler, 1994). Growing evidence demonstrates that NO uses this pathway to regulate the activities of ion channels, receptors, enzymes, and transcription factors (Stamler, 1994). NO-induced S-nitrosylation of thiols is dependent on the availability of oxygen, superoxide anion or transition metal and results in the formation of S-nitrosothiols (Stamler, 1994). S-nitrosylation of proteins regulates their activity by several mechanisms, including post-translational modification of a single thiol residue (Lipton et al., 1993; Stamler et al., 1992), conformational change of the S-nitrosylated protein (Bolotina et al., 1994), and modification of thiol groups within the active site of an enzyme (Dimmeler et al., 1992; Zhang and Snyder, 1992). For instance, NO induces conformational change and stimulates guanidine nucleotide exchange on p21<sup>ras</sup> by S-nitrosylation of a single cysteine residue (C118) (Lander et al., 1997; Lander et al., 1995). Activation of the p21<sup>ras</sup> results in the activation of the MAP kinase pathway (Lander et al., 1996). Formation of S-nitroso-proteins may also play a role in NO storage and transport. For instance, S-nitrosylation of the  $\beta$ 93 cysteine of the hemoglobin occurs in the lungs, where hemoglobin is in the oxygenated state (Jia et al., 1996; Stamler et al., 1992). In the microcirculation, the deoxygenation of hemoglobin triggers NO release (Jia et al., 1996; Stamler et al., 1992). S-nitroso-hemoglobin may play a role in NO storage or transport and may be involved in the control of vascular tone.

### **1.2.3 Free Radicals**

NO has several potential toxic mechanisms, but actual toxicity is highly dependent on NO concentration and the microenvironment in which it is produced. For instance, NO reacts with a number of radicals including hydroxyl, lipid and, superoxide radicals (Beckman and Koppenol, 1996; Lane and Gross,

1999). The reaction of NO with superoxide forms peroxynitrite (Beckman and Koppenol, 1996; Lane and Gross, 1999) and may account for much of the cytotoxic potential of NO (Beckman et al., 1990; Beckman and Koppenol, 1996). Peroxynitrite is a potent oxidant and nitrating agent with a very short half-life (Beckman and Koppenol, 1996). Some of the target molecules of peroxynitrite include membrane lipid (Beckman and Koppenol, 1996; Lane and Gross, 1999), protein sulfhydryl groups (Radi et al., 1991), DNA (Wink et al., 1991), and antioxidants (Beckman and Koppenol, 1996; Lane and Gross, 1999). Peroxynitrite also catalyzes nitration of the tyrosine residues in proteins (Beckman and Koppenol, 1996). Nitration of tyrosine residues has been detected in tissues under different pathological conditions such as atherosclerosis (Beckmann et al., 1994) and hypertension (Bosse and Bachmann, 1997). Several consequences of protein nitration are foreseeable: 1) inhibition of tyrosine phosphorylation (Martin et al., 1990) or conversely, mimicry of tyrosine phosphorylation (Berlett et al., 1996), 2) alteration of protein conformation, and 3) "tagging" of the protein for proteolysis. Nonetheless, biological consequences of the reaction between NO and superoxide remain controversial and are likely to depend on the system studied. For example, the reaction between NO and superoxide prevents oxygen radical-mediated cellular injury in Chinese hamster lung fibroblasts (Wink et al., 1993).

The probability of reaction between NO and superoxide will depend on cellular NO concentration and the rate of the reaction. At low NO concentrations, reaction with cellular superoxide is unfavourable because superoxide dismutase, a scavenger of superoxide, reacts with superoxide at higher rates (Beckman and Koppenol, 1996). Only in pathological conditions, in which NO production is markedly increased, does a reaction with superoxide become favourable (Beckman and Koppenol, 1996).

#### **1.2.4 Targeting of NO Signaling**

As previously mentioned, the cell signal resulting from NO generation will depend on the targets available in the vicinity, their concentration and the rate of reaction with NO. Targeting of NOS to distinct cellular compartments confers specificity to NO signaling. For instance, post-translational modification of eNOS by palmitoylation and myristoylation targets the enzyme to the caveolae (Feron et al., 1998; Garcia-Cardena et al., 1997). Neuronal NOS also interacts with caveolae scaffold proteins (Venema et al., 1997). The caveolae may facilitate eNOS and nNOS interaction with signaling pathways such as the G proteins

and protein kinases. In addition, the PDZ domain targets nNOS to specific areas of the cell where it may interact with signaling molecules and ion channels (Brenman et al., 1996; Brenman et al., 1995).

In summary, at physiological levels, NO activates the sGC/cGMP pathway and induces S-nitrosylation of various intracellular proteins. Targeting of NOS isoforms to specific cellular areas confers specificity to NO signaling by localizing NO generation, and determines the identity of NO targets. At higher concentrations, NO undergoes radical reactions to form peroxynitrite and may be an important mediator of cellular injury.

## 1.3 NO Synthesis in the Kidney

### 1.3.1 NO Synthesis in the Cortex and the Medulla

The methodology for direct measurement of NO synthesis in the kidney is currently limited. Urinary  $\text{NO}_2^-/\text{NO}_3^-$  has been used as an estimate of renal NO production (Shultz and Tolins, 1993). This assay is of limited use for three main reasons. First, plasma  $\text{NO}_2^-/\text{NO}_3^-$  is freely filtered by the glomerulus, and therefore urinary  $\text{NO}_2^-/\text{NO}_3^-$  may originate from both the systemic circulation and renal NO production (Zou and Cowley, 1997). Second, renal tubules both reabsorb and secrete  $\text{NO}_2^-/\text{NO}_3^-$ , which may provide an inaccurate estimate of NO synthesis (Zou and Cowley, 1997). Third, the  $\text{NO}_2^-/\text{NO}_3^-$  assay does not distinguish between regional differences in NO production in the (Zou and Cowley, 1997) kidney. McKee et al. measured the  $\text{Ca}^{+2}$ /calmodulin-dependent conversion of  $^3\text{H}$ -L-arginine to  $^3\text{H}$ -L-citrulline in kidney tissue extracts to assess NOS activity. These measurements revealed that both cortex and medulla (**Figure 1.4**) lysates have considerable NOS activity (McKee et al., 1994). In addition, NOS activity in the medulla was about three-fold higher than that in the cortex and was comparable to the activity measured in the highly NOS enriched cerebellum (McKee et al., 1994). However, *in vitro* measurements of NOS activity may not provide an accurate estimate of NO production since the assay is performed in the presence of excess substrate and cofactor concentrations which may not be relevant *in vivo*. Recently, Zou et al. developed a spectrophotometric technique based on the rapid oxidation of ferrous oxyhemoglobin to methemoglobin by NO in combination with microdialysis to measure NO levels in the interstitial fluid of

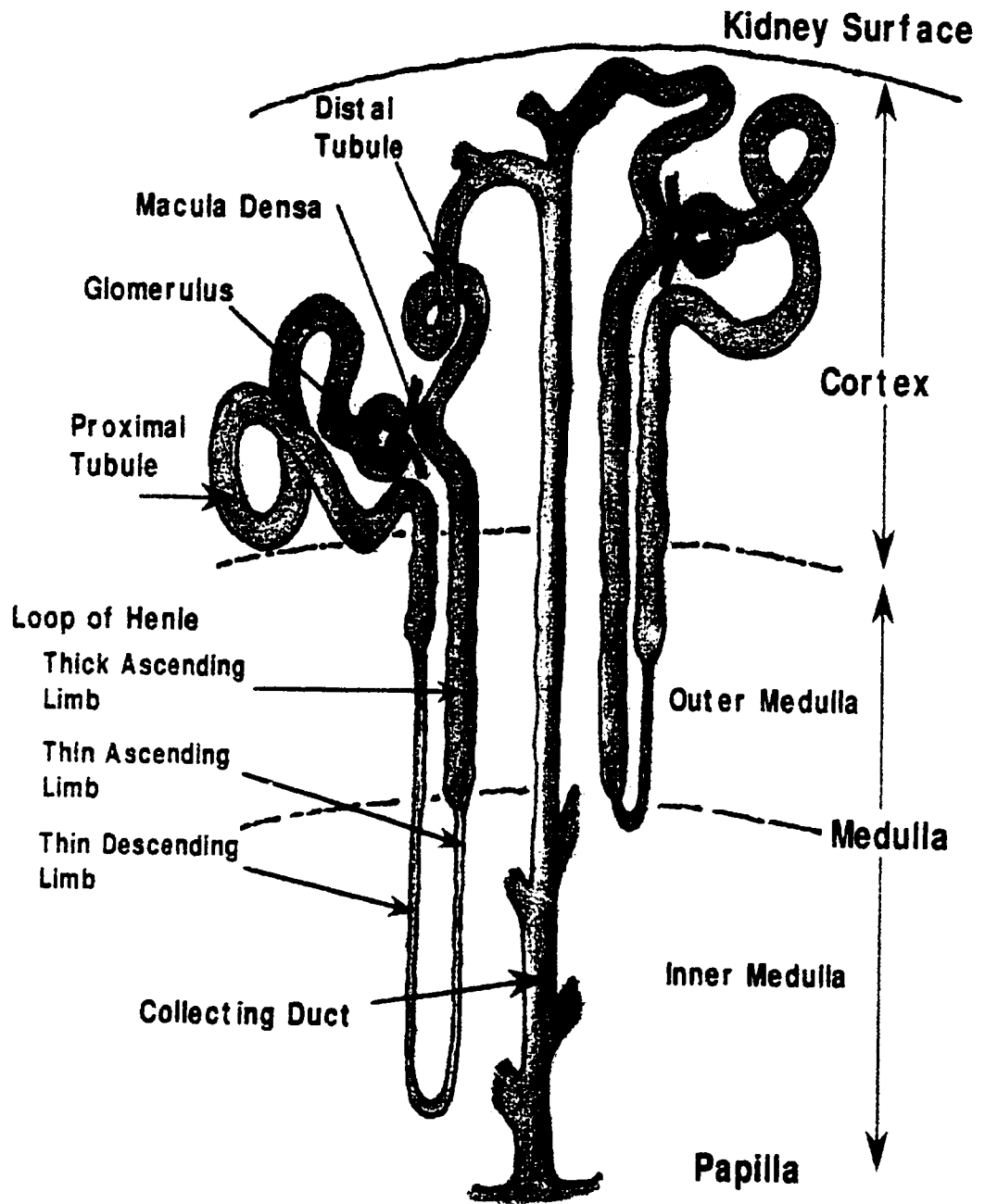
renal cortex and medulla (Zou and Cowley, 1997). This study confirmed that NO concentration in the medullary interstitial fluid (57 nmol/L) was higher than that in the cortex (31 nmol/L) (Zou and Cowley, 1997). Amperometric and voltametric microprobes have also been developed to measure NO concentrations in tissues and single cells (Malinski and Taha, 1992; Shibuki and Okada, 1991). These probes are still relatively insensitive and have never been used in the kidney. Taken together, these studies strongly suggest that NO is produced in the kidney and that regional differences may exist in the quantities of NO produced along the nephron.

### **1.3.2 Localization of NOS in the Kidney**

**Neuronal NOS.** In early studies, nNOS protein was only detected in the cortex (See **Figure 1.4** which depicts the structure of the nephron). By immunohistochemistry nNOS was detected in the macula densa (Bachmann et al., 1995; Wilcox et al., 1992), the efferent arterioles, the glomerular visceral epithelium, and in perivascular nerves surrounding the arcuate and interlobular arteries (Bachmann et al., 1995). Although by RT-PCR the highest levels of nNOS mRNA were detected in the medullary collecting ducts and the thin descending limb of Henle (Terada et al., 1992), the immunohistochemical techniques failed to detect nNOS protein in these tubules (Bachmann et al., 1995). These studies led researchers to conclude that the renal medulla did not express significant levels of nNOS protein and that the principal site on nNOS expression in the kidney was the macula densa in the cortex.

**Endothelial NOS.** Some controversy exists as to the localization of eNOS in the kidney. This isoform is predominantly expressed in endothelial cells and there is no doubt that eNOS mRNA and protein are expressed in the renal vasculature. The immunoreactivity for eNOS protein was detected in the endothelium of intrarenal arteries, the glomerular capillaries, the afferent and efferent arterioles, and the medullary vasa recta (Bachmann et al., 1995; Tojo et al., 1997). However, it is also possible that eNOS is present in renal tubules. Using RT-PCR Ujiie et al. detected eNOS mRNA in the proximal tubule (PT), and the collecting ducts, albeit inconsistently (Ujiie et al., 1994). Yet, LLC-PK1 cells derived from the porcine proximal tubule express eNOS (Tracey et al., 1994).

**Figure 1.4: The nephron.** This picture depicts the various segments of the nephron and the division of the kidney into the cortical, outer medullary and inner medullary regions. This picture is adapted from Schnermann and Sayegh (Schnermann and Sayegh, 1998).



**Inducible NOS.** In most tissues, iNOS isoform expression occurs only after induction by cytokines and LPS. However, RT-PCR and *in situ* hybridization studies have detected constitutive expression of iNOS mRNA in the kidney (Ahn et al., 1994; Mohaupt et al., 1994). These studies showed that iNOS mRNA is tonically expressed along the PT, thick ascending limb (TAL), distal convoluted tubule (DCT), cortical collecting duct (CCD) and inner medullary collecting duct (IMCD) (**Figure 1.4**) of normal rats (Ahn et al., 1994). Furthermore, iNOS mRNA was identified in the glomerulus, and the interlobular and arcuate arteries of the normal rat kidney (Mohaupt et al., 1994). Although the mRNA for iNOS appears to be tonically expressed along the nephron and the renal vasculature, detection of iNOS protein in the unstimulated kidney is problematic. The commercially available iNOS antibodies are relatively non-specific and yield inconsistent results in the kidney (Tojo et al., 1997). Using their own antibody to iNOS, Tojo et al. demonstrated immunostaining for iNOS in the intercalated cells of the CCD, localized to the apical region of the cells (Tojo et al., 1994) and also in TAL, DCT and afferent arterioles (Tojo et al., 1994). *In situ* hybridization on kidneys from rats treated with LPS demonstrated strong induction of iNOS mRNA in glomerular mesangial cells, and inner medullary interstitial cells (Ahn et al., 1994). In cultured cells, treatment with cytokines or LPS induced iNOS protein expression in PT (Guzman et al., 1995), and IMCD cells (Mohaupt et al., 1995). These studies demonstrated that both the renal vasculature and renal tubules have the capacity to express iNOS.

### **1.3.3 Localization of the Components of the NO/cGMP Pathway in the Kidney.**

The components of the NO/cGMP signaling pathway in the kidney demonstrate a differential distribution, indicating that NO signaling may vary from one region of the kidney to another. The mRNA for sGC is highly expressed in glomeruli, PT, CCD and at lower levels in medullary TAL (mTAL), IMCD and the vasculature (Terada et al., 1992), indicating that all regions of the kidney have the capacity to generate cGMP. The distribution of the cGKs in the kidney however, follows a very specific pattern. cGKI is a cytosolic protein localized in renal vascular smooth muscle cells, mesangial cells and interstitial cells, and is not expressed in renal tubules (Gambaryan et al., 1996). cGKII on the other hand, is membrane-associated and found in the brush border of the PT and in the juxtaglomerular renin-secreting

cells (Gambaryan et al., 1996). In the juxtaglomerular cells, cGKII is associated with renin-containing vesicles (Gambaryan et al., 1996), suggesting that it may play a role in the regulation of renin secretion.

The distribution of phosphodiesterases in the kidney has not been studied in detail. PDE4 is a widely expressed isoform and may be expressed in the kidney, whereas both PDE3 and 5 are found at high levels in vascular smooth muscle cells (Beavo, 1995). PDE3 is also expressed in the juxtaglomerular cells (Kurtz et al., 1998).

## **1.4 The Role of NO in Renal Hemodynamics and Juxtaglomerular Apparatus Function**

### **1.4.1 Regulation of Renal Hemodynamics by NO**

**Glomerular filtration rate (GFR).** The plasma entering the glomerulus by the afferent arteriole is filtered by the glomerular capillaries, and then exits by the efferent arteriole (**Figure 1.5**). The difference between the capillary ( $P_{GC}$ ) and the Bowman's space ( $P_B$ ) hydrostatic pressures drives the filtration of plasma, whereas the difference between the capillary ( $\pi_{GC}$ ) and Bowman's space ( $\pi_B$ ) oncotic pressures opposes plasma filtration (Schnermann and Sayegh, 1998). The characteristics of the glomerular capillary membrane also determine GFR. The glomerular capillary permeability and the filtration surface are not easily measured independently, but the product of these two determinants can be measured and is called the glomerular filtration coefficient ( $K_f$ ) (Schnermann and Sayegh, 1998). GFR can therefore be calculated as follows:

$$GFR = K_f \times (P_{GC} - P_B) - (\pi_{GC} - \pi_B) \text{ (Schnermann and Sayegh, 1998)}$$

Alterations in the resistance of the afferent and efferent arterioles regulate GFR and renal plasma flow (RPF). An increase in the afferent arteriole resistance decreases the glomerular capillary hydrostatic pressure and RPF and results in decreased GFR (Schnermann and Sayegh, 1998). Conversely, increased efferent arteriole tone increases glomerular capillary pressure and increases GFR despite a reduced RPF (Schnermann and Sayegh, 1998).

***Effect of acute and chronic NOS inhibition.*** Administration of NOS inhibitors increases mean arterial pressure (MAP), renal vascular resistance, and decreases both RPF and GFR in rats (Baylis et al., 1990) and in human volunteers (Bech et al., 1998; Bech et al., 1996; Haynes et al., 1997). Larger reductions in RPF than in GFR result in increased filtration fraction (Baylis et al., 1990; Bech et al., 1996). In addition, NOS inhibition is accompanied by a significant decrease in urinary Na<sup>+</sup> excretion (Baylis et al., 1990; Bech et al., 1996). Micropuncture studies in rats demonstrated that the decrease in single nephron plasma flow (snPF) after NOS blockade is due to an increase in afferent and efferent arteriole resistance (De Nicola et al., 1992; Zatz and de Nucci, 1991). Both in vivo and in vitro studies show that K<sub>f</sub> also decreases during NO synthesis inhibition, due to mesangial cell contraction (Zatz and de Nucci, 1991), reducing the filtration area of the glomerular endothelium. These studies have been corroborated in the isolated perfused kidney in order to avoid effects of neurohumoral factors. In these studies infusion of NOS inhibitor increased the resistances of both afferent and efferent arterioles, increased filtration fraction and decreased the K<sub>f</sub> (Radermacher et al., 1992). However, in isolated perfused cortical arterioles attached to a glomerulus, NO preferentially influences afferent arteriolar resistance, with a minimal effect on the efferent arteriole (Ito et al., 1993; Ito et al., 1991).

Prolonged NOS inhibition (2 months) causes a significant increase in the systemic blood pressure that persists for as long as NOS inhibitor is administered. Prolonged NOS inhibition causes a significant reduction in GFR and RPF and eventually leads to the development focal glomerulosclerosis, vascular damage and proteinuria (Baylis et al., 1992; Fujihara et al., 1995).

In summary, NOS blockade produces profound changes in both systemic and renal hemodynamics which include an increase in systemic blood pressure, an increase in the afferent arteriole resistance, a decrease in K<sub>f</sub> and variable effects on efferent arteriole resistance. Prolonged NO inhibition results in severe renal damage.

#### **1.4.2 The Role of NO in the Function of the Juxtaglomerular apparatus**

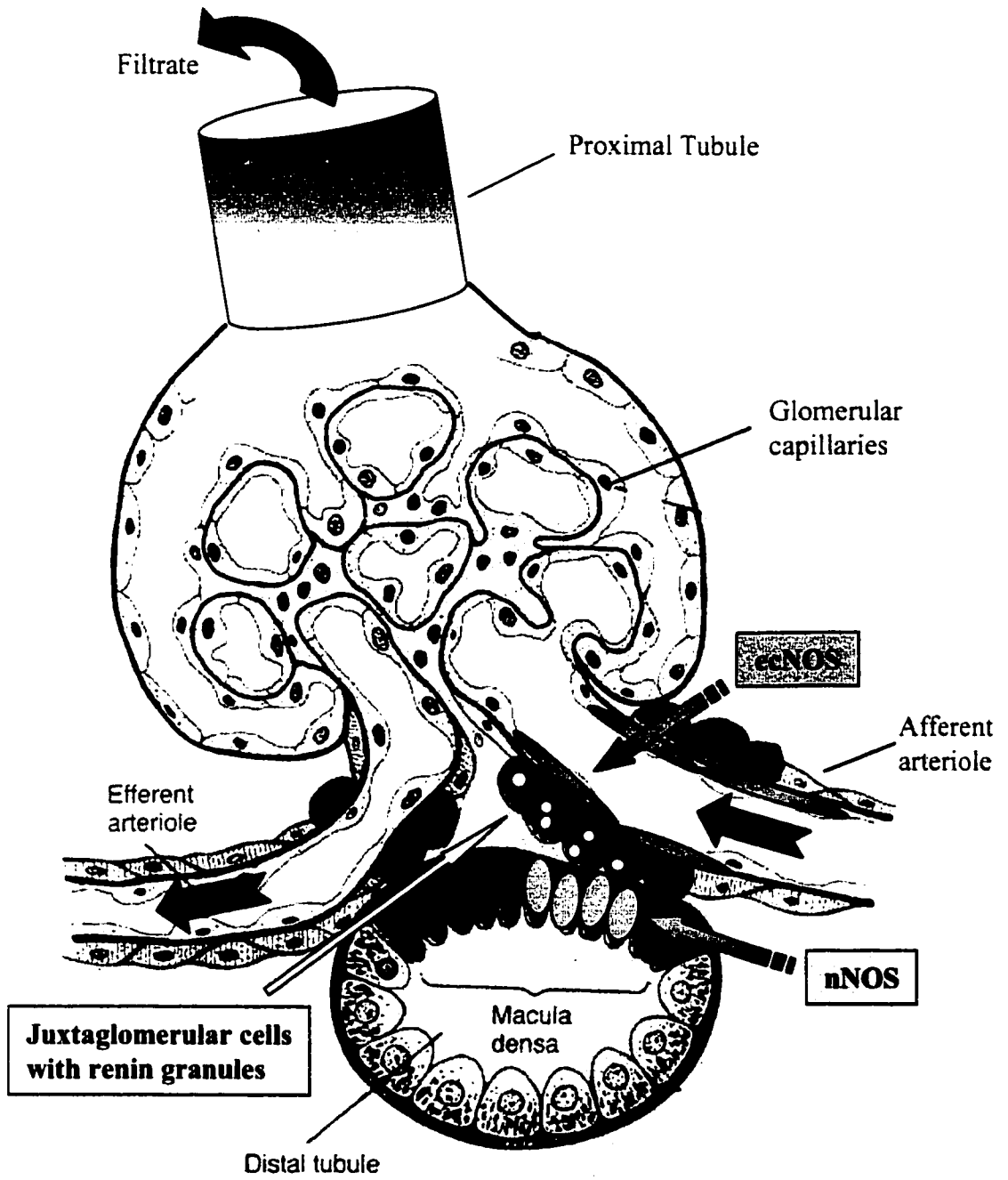
***The juxtaglomerular apparatus (JGA).*** The initial portion of the distal tubule, immediately after the upper end of the thick ascending limb of the loop of Henle, passes between the afferent and efferent arterioles at the site where these vessels enter (the afferent arteriole) and leave (the efferent arteriole) the

glomerulus (**Figure 1.5**). The epithelial cells of the distal tubule that are in contact with the afferent arteriole are called the macula densa cells (Schnermann, 1998). The smooth muscle cells of the afferent arteriole that are in contact with the macula densa cells are the renin secreting cells and are called the juxtaglomerular cells (Schnermann, 1998). The whole complex of the macula densa and juxtaglomerular cells is called the juxtaglomerular apparatus (Schnermann, 1998). The two main functions of the JGA are the regulation of tubuloglomerular feedback (TGF) and renin secretion.

***Role of NO in the modulation of TGF.*** The TGF mechanism prevents fluctuations in GFR in response to fast and random perturbations in arterial blood pressure (Schnermann, 1998). Thus, the TGF system renders  $\text{Na}^+$  excretion relatively independent of the minute-to-minute fluctuations in blood pressure, unrelated to body  $\text{Na}^+$  balance (Schnermann, 1998). This is important because even a 5% increase in GFR may cause excess loss of solutes and water in the urine. Activation of the TGF mechanism is dependent on  $\text{Na}^+$  reabsorption by the macula densa. For instance, when GFR increases,  $\text{NaCl}$  delivery to the distal tubule increases and stimulates reabsorption of  $\text{NaCl}$  by the macula densa cells (Schnermann, 1998). Increased  $\text{NaCl}$  reabsorption triggers a chain of events that culminates in the constriction of the afferent arteriole and reduction of GFR back to normal levels (Schnermann, 1998). The signaling pathway that connects increased  $\text{NaCl}$  transport in the macula densa to the afferent arteriole constriction is presently unknown, although both Ang II and adenosine have been proposed as mediators (Schnermann, 1998).

NO is a modulator of the TGF response. Using micropuncture, Wilcox et al. demonstrated that inhibition of NO synthesis during perfusion of the loop of Henle significantly decreases tubular stop flow pressure (PSF), an index of GFR (Wilcox et al., 1992). Indeed, further studies demonstrated that NO reduces afferent arteriole constriction triggered by TGF (Ito and Ren, 1993). These studies suggest that NO counteracts TGF-induced afferent arteriole constriction. This effect may be either direct, by NO-mediated vasodilatation of the afferent arteriole, or indirect, by NO-mediated inhibition of macula densa  $\text{Na}^+$  reabsorption, thereby diminishing the signal for TGF activation.

**Figure 1.5: The juxtaglomerular apparatus.** The JGA is composed of the macula densa and of the renin-containing juxtaglomerular cells. Neuronal NOS is expressed in the macula densa cells whereas ecNOS is expressed in the afferent arteriole. This figure is adapted from Guyton (Guyton, 1991).



Adapted from Guyton et al., 1991.

The identity of the NOS enzyme that participates in the TGF response is a subject of controversy. Due to their localization, both ecNOS and nNOS may be involved (**Figure 1.5**). With regards to nNOS, acute treatment with a relatively specific inhibitor of nNOS, 7-nitro-indazole (7NI), increased TGF sensitivity and resulted in the significant reduction of GFR, suggesting that nNOS may be involved in the modulation of TGF (Ollerstam et al., 1997). Furthermore, prolonged treatment with 7NI resulted in the development of hypertension (Ollerstam et al., 1997). Although studies have not been performed to examine the role of ecNOS in TGF modulation, it is of interest that ecNOS is expressed in the endothelial cells of the afferent arteriole (Bachmann et al., 1995) and that its activity is upregulated by shear stress (Ranjan et al., 1995). Until highly specific NOS isoform inhibitors are developed, the identity of NOS isoform involved in TGF modulation may not be identified with certainty. Furthermore, studies on NOS knockout mice may be useful to clarify the role of NO in TGF modulation.

Taken together, these studies indicate that nNOS expressed in the macula densa may be an important modulator of the TGF response. Inhibition of nNOS activity impairs proper regulation of GFR and results in solute and water retention and development of hypertension.

***Role of NO in regulating renin secretion.*** Renin, an enzyme secreted by the granular cells of the JGA, catalyzes the limiting step in the generation of angiotensin II (Ang II) (**Figure 1.5**). The renin-Ang II system (RAS) is a powerful mechanism for regulating body fluid volume and controlling blood pressure (Hall et al., 1999). Ang II is a potent vasoconstrictor and stimulator of  $\text{Na}^+$  and water reabsorption by the kidney (Hall et al., 1999). Consequently, renin secretion is activated in conditions associated with acute volume depletion and hypotension and inhibited by body volume expansion and hypertension (Hall et al., 1999). There are three main signaling mechanisms involved in stimulating renin secretion. The first involves increased sympathetic activity, the second decreased afferent arteriolar pressure, and the third involves the JGA (Schnermann and Sayegh, 1998). In response to decreased GFR, the delivery of NaCl to the macula densa decreases and triggers renin release (Schnermann, 1998). The signaling mechanism that links uptake of NaCl by the macula densa to renin secretion is poorly understood.

Inhibition of NO synthesis in humans and animals reduces both plasma renin activity and renal renin mRNA expression (Schrickler et al., 1995; Wagner et al., 1998). This effect is further potentiated by prior stimulation of renin synthesis by Ang II antagonists (Schrickler et al., 1995). These studies suggest

that NO has a tonic stimulatory effect on renin secretion *in vivo*. However, studies in intact animals are difficult to interpret due to variations in blood pressure induced by NO blockers, which by itself regulates renin release (Wagner et al., 1998). To avoid interference of the cardiovascular reflexes, studies have also been performed on simpler systems such as isolated perfused kidneys, isolated perfused JGA, and granular cells in culture. In isolated perfused kidneys endogenous NO stimulated renin release, confirming data obtained *in vivo* (Scholz and Kurtz, 1993). However, in isolated granular cells NO inhibited renin secretion in a cGMP-dependant fashion (Greenberg et al., 1995; Schricker and Kurtz, 1993). Studies performed on isolated perfused JGA provided an even more complex picture of the NO effect. In this model, renin secretion is stimulated when NO formation in the macula densa is stimulated and inhibited when NO is applied directly to the juxtaglomerular cells (He et al., 1995).

These studies imply a dual effect of NO on renin secretion. Coincidentally, two isoforms of NOS are present in the vicinity of the renin-secreting granular cells. The ecNOS is expressed in the endothelial cells of the afferent arteriole (Bachmann et al., 1995) and could mediate the inhibitory effect of NO on renin secretion. Neuronal NOS on the other hand, is expressed in the macula densa (Bachmann et al., 1995) and could mediate the stimulatory effect on renin secretion. Indeed, selective nNOS blockade inhibited furosemide-stimulated renin secretion *in vivo* (Beierwaltes, 1995).

The regulation of renin secretion by NO occurs by two separate NO/cGMP signaling pathways. In the isolated perfused kidney, NO stimulated renin secretion by cGMP-mediated inhibition of PDE3 (Kurtz et al., 1998). On the other hand, in mice with targeted disruption of cGKII gene, renin levels were significantly elevated, suggesting that activation of cGKII inhibits renin secretion (Gambaryan et al., 1998; Wagner et al., 1998).

In summary, these studies indicate that NO exerts a dual effect on renin secretion, which may be due to the actions of two separate isoforms of NOS and activation of two separate NO/cGMP signaling pathways. Further studies are required to clarify the role of the different NOS isoforms in the regulation of renin secretion.

## 1.5 The Role of NO in the Regulation of Na<sup>+</sup> transport

### 1.5.1 Regulation of Na<sup>+</sup> and water reabsorption by the kidney

**Pressure Natriuresis.** One of the many functions of the kidney is to regulate extracellular volume composition by controlling Na<sup>+</sup> and water excretion. For this reason the kidney plays a key role in the long-term control of arterial blood pressure. Guyton et al. demonstrated that the kidney precisely adjusts Na<sup>+</sup> and water excretion in response to changes in renal perfusion pressure, in a process known as pressure-natriuresis (Guyton, 1991). In response to increased arterial blood pressure, the kidney excretes more Na<sup>+</sup> and water than is entering the body, thereby decreasing arterial blood pressure to normal levels (Guyton, 1991). Conversely, when the blood pressure falls the kidney excretes less Na<sup>+</sup> and water than is entering the body, hence the arterial blood pressure increases back to normal (Guyton, 1991). In the end, to maintain constant extracellular fluid composition the intake of both Na<sup>+</sup> and water must be matched by an equal amount of Na<sup>+</sup> and water excretion. An important feature of pressure natriuresis is that it has infinite feedback gain (Guyton, 1991). This means that pressure natriuresis operates until the pressure returns to its initial set point. Guyton et al., further proposed that hypertension could only develop if renal handling of salt and water was impaired. Indeed, in all forms of hypertension studied thus far, there is a shift of renal pressure natriuresis that requires increased arterial pressure to maintain sodium and water balance (Cowley and Roman, 1996; Lifton, 1996). In addition, when kidneys from hypertensive rats were transplanted into normotensive rats, the normotensive rats developed high blood pressure (Lifton, 1996). Conversely, when the kidneys from normotensive rats were transplanted into hypertensive rats, the hypertension in these rats was abrogated (Lifton, 1996). These experiments have been conducted on Dahl salt-sensitive and salt-resistant rats as well as on spontaneously hypertensive rats (Lifton, 1996). These studies suggest that a defect in the kidney may be involved in the development of hypertension.

**Mechanisms of renal Na<sup>+</sup> reabsorption.** Renal hemodynamic effects and renal tubular reabsorption determine Na<sup>+</sup> and water excretion in the urine. The initial step in urine formation is the filtration of plasma through the glomerular capillaries (Schnermann and Sayegh, 1998). The general mechanism of Na<sup>+</sup> reabsorption along the nephron is based on the generation of the electrochemical

gradient by the basolateral  $\text{Na}^+/\text{K}^+$  ATPase that allows  $\text{Na}^+$  to be transported from the tubular lumen to the interstitium (Schnermann and Sayegh, 1998). With the exception of the collecting duct (CD),  $\text{Na}^+$  is co-transported or counter-transported with other molecules (glucose, amino acids,  $\text{Cl}^-$  or  $\text{H}^+$ ) which are carried against their concentration gradients (Schnermann and Sayegh, 1998). These transporters, also called secondary transporters, are expressed on the apical membranes of renal tubules and localization varies along the nephron (Schnermann and Sayegh, 1998).  $\text{Na}^+$  and water are mainly reabsorbed in the proximal tubule (PT), and the thick ascending limb (TAL), whereas the distal convoluted tubule (DCT) and the collecting duct (CD) are involved in the fine-tuning of  $\text{Na}^+$  and water reabsorption for balance purposes (Gamba, 1999).

In the PT, approximately 65% of filtered NaCl and water is reabsorbed and returned to the circulation (Schnermann and Sayegh, 1998). Although several transporter proteins mediate  $\text{Na}^+$  reabsorption in the PT, the apical  $\text{Na}^+/\text{H}^+$  exchanger (NHE3) reabsorbs the majority of  $\text{Na}^+$  and  $\text{HCO}_3^-$  (Schultheis et al., 1998). NHE3 belongs to a family of transporters encoded by separate genes that also includes NHE1, NHE2, NHE4 and NHE5 (Wakabayashi et al., 1997). Intracellular pH, growth factors, hormones and increased osmolarity rapidly activate NHE3 (Wakabayashi et al., 1997). The cytoplasmic  $\text{H}^+$  is an allosteric modifier of the exchanger and it greatly stimulates NHE3 activity (Wakabayashi et al., 1997). In addition, NHE3 is an important target for Ang II (Geibel et al., 1990). In volume contracted states, Ang II directly activates NHE3 to increase  $\text{Na}^+$  reabsorption (Geibel et al., 1990). Another characteristic of NHE3 is that it is inhibited by the diuretic drug amiloride, and its derivatives (Wakabayashi et al., 1997). A recent study demonstrated that mice with a targeted deletion of the NHE3 gene are hypotensive and mildly acidotic (Schultheis et al., 1998), demonstrating the importance of NHE3 in blood pressure and pH regulation.

The TAL reabsorbs approximately 15 to 20% of NaCl and regulates  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  excretion in the urine (Schnermann and Sayegh, 1998). In addition, the TAL plays an essential role in the production and maintenance of renal medullary hypertonicity, as it reabsorbs  $\text{Na}^+$  but is impermeable to water (Gamba, 1999). The major pathway for  $\text{Na}^+$  reabsorption in the TAL is the furosemide-sensitive  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  symporter (Gamba, 1999). The symporter is encoded by two separate genes: BSC1 encodes the kidney specific symporter (Gamba et al., 1994; Payne and Forbush, 1994), whereas BSC2 encodes the

ubiquitously expressed  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  (Delpire et al., 1994; Xu et al., 1994). BSC1 is alternatively spliced to form 6 different isoforms and at least three of these are expressed in the TAL (Mount et al., 1999). The functional significance of the alternatively spliced isoforms of BSC1 is unknown. Vasopressin (AVP) is of great importance in the regulation of  $\text{Na}^+$  reabsorption in the TAL since it stimulates the activity of both the  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  symporter and of the apically expressed  $\text{K}^+$  channel (Gamba, 1999). Increased  $\text{K}^+$  secretion generates a lumen-positive potential which enhances  $\text{Na}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  uptake by the paracellular pathway (Gamba, 1999). Mutations of the BSC1 gene occur in patients with Bartter's syndrome, a disease characterized by metabolic alkalosis, hypokalemia, severe salt wasting and hypercalciuria (Simon et al., 1996).

The DCT reabsorbs 5% to 7% of filtered  $\text{Na}^+$  and also participates in the urinary concentration mechanism (Schnermann and Sayegh, 1998). In the DCT  $\text{Na}^+$  is reabsorbed by a thiazide-sensitive  $\text{Na}^+/\text{Cl}^-$  symporter, which is encoded by the TSC1 gene (Gamba et al., 1993). Missense mutations, premature termination codons and splice site mutations in the  $\text{Na}^+/\text{Cl}^-$  gene have been identified in patients with Gitelman's syndrome, which is characterized by renal salt wasting, hypokalemic alkalosis, hypocalciuria and hypomagnesemia (Simon et al., 1996).

The CD reabsorbs 1% to 2% of  $\text{Na}^+$  and reabsorbs water, however the reabsorptive capacity of the CD varies depending on the presence of aldosterone and AVP (Schnermann and Sayegh, 1998). An electrogenic and amiloride-sensitive process mediates  $\text{Na}^+$  reabsorption in the CCD, outer medullary collecting duct (OMCD) and IMCD (Gamba, 1999). Studies have shown that the expression of the amiloride-sensitive epithelial  $\text{Na}^+$  channel (ENaC) is restricted to the distal part of the nephron which includes the CCD, OMCD, and IMCD (Duc et al., 1994). In particular, ENaC is highly expressed in the apical membranes of the IMCD and may be the primary route of  $\text{Na}^+$  reabsorption in this nephron segment (Volk et al., 1995). Aldosterone is a mineralocorticoid, which is secreted from the adrenal gland in response to angiotensin II stimulation. Aldosterone interacts with mineralocorticoid receptors in the CD, leading to increased ENaC activity and elevated salt and water reabsorption. A number of human diseases have been linked to malfunction or mutations in ENaC, including Liddle's syndrome, and pseudohypoaldosteronism type I (Rossier, 1997). Liddle's syndrome is an autosomal dominant form of inherited human arterial hypertension, characterized by an early onset of severe hypertension and salt

sensitivity (Rossier, 1997). Liddle's syndrome has been genetically linked to mutations that cause truncation of  $\beta$  and  $\gamma$  ENaC subunits and result in elevated ENaC activity due to both an increase in the channel number in the apical membrane and an increase in the mean open probability of each channel (Firsov et al., 1996; Schild et al., 1995; Snyder et al., 1995).

### **1.5.2 Regulation of $\text{Na}^+$ excretion by NO.**

***Role of NO in pressure natriuresis.*** In animals and humans treated with small doses of L-NAME, urine volume and  $\text{Na}^+$  excretion decrease despite the absence of alterations in mean arterial pressure or GFR (Bech et al., 1996; Lahera et al., 1991; Shultz and Tolins, 1993). The anti-natriuretic and anti-diuretic effect of L-NAME is abrogated by exogenous cGMP (Lahera et al., 1993). These studies suggest that NO inhibits renal  $\text{Na}^+$  reabsorption by stimulating the production of cGMP. Studies were also performed to investigate the role of NO in the process of pressure natriuresis. Intrarenal infusion of L-NAME abolished urinary  $\text{Na}^+$  excretion in response to elevated perfusion pressure, demonstrating that inhibition of NO synthesis impairs pressure natriuresis (Salom et al., 1992). Furthermore, concomitant administration of saline load and L-NAME induced a significant increase in arterial pressure, secondary to  $\text{Na}^+$  retention (Salazar et al., 1993). Taken together these studies indicate that NO synthesis is important in the regulation of renal  $\text{Na}^+$  excretion and may be one of the components of pressure natriuresis.

***Effect of NO on renal medullary blood flow and  $\text{Na}^+$  excretion.*** Changes in medullary blood flow have been implicated in the regulation of renal  $\text{Na}^+$  reabsorption and pressure natriuresis (Cowley et al., 1992). A decrease in the medullary blood flow reduces the interstitial hydrostatic pressure and consequently increases  $\text{Na}^+$  transport in the mTAL, OMCD and IMCD (Cowley et al., 1992; Knox et al., 1980). Conversely, an increase in the medullary blood flow increases the interstitial hydrostatic pressure and reduces  $\text{Na}^+$  reabsorption in the mTAL, OMCD and IMCD (Cowley et al., 1992; Knox et al., 1980).

Numerous studies show that the medulla produces the highest levels of NO in the kidney (McKee et al., 1994; Zou and Cowley, 1997). The role of NO in the medulla has been investigated by infusion of NOS inhibitors, at concentrations that do not affect either the systemic or cortical blood flow, directly into the medullary interstitial space. These studies demonstrate that inhibition of NO production in the medulla is associated with a significant decrease in the medullary blood flow (Fenoy et al., 1995; Mattson et al.,

1994; Mattson et al., 1992). Furthermore, when animals are simultaneously fed a high  $\text{Na}^+$  diet, the inhibition of NO synthesis in the medulla is associated with  $\text{Na}^+$  retention and development of hypertension (Mattson et al., 1994). To determine which isoform of NOS is involved in the regulation of medullary blood flow, Mattson et al., infused antisense to nNOS directly into the inner medulla (Mattson and Bellehumeur, 1996). This study showed that in animals fed a high salt diet, inhibition of inner medullary nNOS resulted in  $\text{Na}^+$  retention and subsequent development of hypertension (Mattson and Bellehumeur, 1996). Hence, these studies suggest that NO generated by the inner medullary nNOS inhibits  $\text{Na}^+$  reabsorption possibly by increasing medullary blood flow and increasing the interstitial hydrostatic pressure. Furthermore, inhibition of NO synthesis in the medulla impairs pressure natriuresis and causes development of hypertension.

***Dietary NaCl regulates renal NO production.*** During high dietary NaCl intake, serum concentration and urinary excretion of the NO decomposition products  $\text{NO}_2^-$  and  $\text{NO}_3^-$  increase, suggesting that NO production in the kidney is increased (Shultz and Tolins, 1993). This suggests that NO production represents an adaptive mechanism that induces vasodilatation and natriuresis, thereby contributing to the maintenance of normal blood pressure during high NaCl intake. The mechanism of NO synthesis regulation by high dietary salt is unclear. However, a number of investigators showed that high dietary NaCl decreases nNOS mRNA and protein abundance in the macula densa (Bosse et al., 1995; Schricker et al., 1996; Singh et al., 1996) and has no effect on eNOS protein expression in the cortex (Mattson and Higgins, 1996). On the other hand, both nNOS and eNOS proteins are upregulated in the medulla of rats fed a high salt diet (Mattson and Higgins, 1996). These studies suggest that the effect of high dietary salt on NOS expression may be isoform and cell-specific.

***Effect of NO on renal  $\text{Na}^+$  transporters.*** The mechanism of renal  $\text{Na}^+$  retention during NOS inhibition is unclear because both vascular and tubular alterations could be involved. Indeed, studies demonstrated that NO inhibits  $\text{Na}^+$  uptake in the PT and the CCD. For instance, in mouse PT cells (MCT) induction of iNOS expression with LPS and IFN inhibits  $\text{Na}^+/\text{K}^+$  ATPase activity (Guzman et al., 1995). This effect is probably mediated by the formation of free radicals since treatment of cells with superoxide dismutase, a radical scavenger, prevented the inhibition (Guzman et al., 1995). In mouse M1 CCD cells cocultured with cow pulmonary artery endothelial cells, stimulation with bradykinin or acetylcholine led to

cGMP generation and inhibition of apical  $\text{Na}^+$  transport (Stoos et al., 1992; Stoos et al., 1994). These studies were repeated on microdissected CCD and showed that NO donors significantly decreased  $\text{Na}^+$  entry at the apical membrane probably by affecting amiloride-sensitive channels (Stoos et al., 1995). NO also reduces AVP-stimulated water permeability in the CCD by stimulating production of cGMP (Garcia et al., 1996). These studies indicate that NO directly inhibits the activities of a number of renal  $\text{Na}^+$  transporters. However, no studies were performed to determine whether NO regulates  $\text{Na}^+$  uptake by the NHE3 transporter in the PT.

### 1.5.3 NO and Hypertension

***NO and salt-sensitive hypertension.*** L. K. Dahl developed two strains of rats from the Sprague-Dawley line, one that is sensitive (DS) and the other resistant (DR) to the hypertensive effects of a high salt diet (Sanders, 1996). The DS and DR rats were further inbred by J. P. Rapp to generate two strains that are each homozygous at 100% of all genetic loci (Sanders, 1996). When fed an 8% NaCl chow DS rats rapidly develop low-renin hypertension but DR rats remain normotensive (Sanders, 1996). Studies also demonstrated that DS rats retain abnormal amounts of  $\text{Na}^+$  during acute NaCl-loading (Sanders, 1996). Furthermore, cross-transplantation of kidneys between DS and DR rats, prevented development of hypertension in DS rats. Thus, genes involved in  $\text{Na}^+$  homeostasis and renal function may be responsible for the development of hypertension in the DS rat (Sanders, 1996).

Impaired NO synthesis with consequent maladaptive renal  $\text{Na}^+$  reabsorption has been implicated in the pathogenesis of salt-sensitive hypertension (Chen and Sanders, 1991; Higashi et al., 1996; Ikeda et al., 1995). Salt-sensitive patients (Higashi et al., 1996) and DS rats (Chen and Sanders, 1993; Hu and Manning, 1995; Simchon et al., 1996) exhibit increased renal vascular resistance and an impaired ability to generate NO during high dietary  $\text{Na}^+$  intake as compared to salt-resistant hypertensive patients and DR rats.

The reason for reduced NO synthesis in the kidneys of DS rats is unknown, but measurement of NOS activity in the kidneys of DS and DR rats revealed that nNOS activity, but not that of eNOS or iNOS, is significantly decreased in the DS rats (Ikeda et al., 1995). In addition, the nNOS and iNOS mRNA expression in the kidneys of salt-sensitive Sabra rats is also decreased as compared to the salt-resistant Sabra rats (Lippoldt et al., 1997). The consequences of reduced nNOS activity include decreased

medullary blood flow and development of hypertension secondary to Na<sup>+</sup> retention. Indeed, Tan et al. demonstrated that inhibition of nNOS in DR rats with 7NI caused salt-sensitive hypertension (Tan et al., 1999) whereas Mattson et al., showed that inhibition of medullary NOS activity decreases medullary blood flow and is associated with increased blood pressure (Mattson et al., 1997).

Studies on TAL tubules and IMCD cells from DS rats revealed that renal segments reabsorbed significantly more Na<sup>+</sup> than the segments obtained from DR rats (Garcia et al., 1999; Husted et al., 1996), perhaps due to reduced NO synthesis in the kidney of DS rats. Furthermore, pressure natriuresis is impaired, and the medullary blood flow reduced in DS rats. Administration of L-arginine, to stimulate NO production, normalizes pressure natriuresis (Hu and Manning, 1995), alters renal tubular transport (Kirchner et al., 1995) and prevents hypertension in the DS rat (Chen and Sanders, 1991; Chen and Sanders, 1993). Interestingly, infusion of L-arginine directly into the medulla also prevents the reduction in the medullary blood flow and hypertension in the DS rats (Miyata and Cowley, 1999; Miyata et al., 1998).

In summary, NO synthesis is impaired in both human and rat salt-sensitive hypertension. The reason for defective NO generation is unknown but both nNOS mRNA expression and activity in the kidney are significantly reduced in DS rats. In addition, infusion of L-arginine to the renal medulla prevents reduction in medullary blood flow and normalizes blood pressure in DS rats, suggesting that the defect may reside in the medullary capacity to synthesize NO.

**NO and chronic renal failure (CRF).** CRF is characterized by the progressive loss of nephrons, associated with increased intraglomerular pressure and with hyperfiltration (Brenner et al., 1982). 5/6 nephrectomy (5/6 Nx) is a rat model of CRF where one kidney and 2/3 of the other are surgically removed. Rats undergoing 5/6 Nx initially demonstrate an adaptive increase in sngFR and snPF and hypertrophy of the remnant kidney (Anderson et al., 1985). Eventually, a reduction of both GFR and RBF ensues, and the 5/6 animals develop severe proteinuria, and glomerulosclerosis. Systemic hypertension develops early in this model (Anderson et al., 1985; Yoshida et al., 1989).

Several studies have suggested that NO production in CRF is significantly reduced. For instance, rats with renal mass reduction (RMR) show reduced urinary excretion of NO metabolites (nitrites/nitrates) and diminished histochemically detectable iNOS and eNOS in the remnant kidney (Aiello et al., 1997). In

addition, accumulation of endogenous NOS inhibitors such as ADMA, has been demonstrated in patients with CRF (Vallance et al., 1992). Administration of L-arginine to rats with CRF increases GFR, reduces proteinuria and preserves renal morphology by increasing NO production (Ashab et al., 1995). Thus, CRF is a condition of low NO production in the kidney probably due to decreased iNOS and ecNOS expression and increased levels of ADMA. Renal nNOS expression, however has not been studied in the rat model of CRF.

## **1.6 Interaction of NO with the Renin-Angiotensin II system (RAS)**

### **1.6.1 The renin-angiotensin II system**

Ang II is an important component of the pressure natriuresis system (Guyton, 1991). During high  $\text{Na}^+$  intake Ang II levels are suppressed which dramatically decreases renal  $\text{Na}^+$  reabsorption (Hall et al., 1999). Conversely, when  $\text{Na}^+$  intake is low Ang II levels increase and greatly stimulate renal  $\text{Na}^+$  uptake (Hall et al., 1999). When RAS functions properly the relationship between arterial pressure and  $\text{Na}^+$  excretion is steep which means that sodium balance is maintained with minimal changes in arterial blood pressure (Guyton, 1991; Hall et al., 1999). Ang II promotes renal  $\text{Na}^+$  reabsorption by both hemodynamic and tubular effects (Hall et al., 1999). Ang II alters both peritubular capillary dynamics and reduces medullary blood flow to increase  $\text{Na}^+$  reabsorption (Hall et al., 1999). In the PT, Ang II stimulates the activities of NHE3,  $\text{Na}^+/\text{K}^+$  ATPase and  $\text{Na}^+/\text{HCO}_3^-$  cotransport (Geibel et al., 1990). Evidence suggests that Ang II also increases the activity of the  $\text{Na}^+/\text{K}^+ / 2\text{Cl}^-$  cotransporter in the medullary TAL (Amlal et al., 1998) and may stimulate  $\text{Na}^+$  reabsorption in the distal nephron (Hall et al., 1999). Ang II acts in concert with the TGF mechanism to maintain GFR in the face of decreased perfusion pressure. Ang II exerts its main constrictor activity on the efferent arteriole resistance. In addition, Ang II enhances TGF sensitivity probably by stimulating macula densa  $\text{Na}^+$  reabsorption (Navar et al., 1996).

## 1.6.2 Interaction of NO and RAS

*Interaction at the vascular level.* At the vascular level NO counteracts vasoconstriction induced by Ang II. Thus, Ang II blockade prevents the renal vasoconstrictor responses to NOS inhibition but does not prevent the systemic hypertension induced by acute NOS blockade (Baylis et al., 1994). Perfusion of afferent and efferent glomerular arterioles attached to the glomerulus stimulated NO production and inhibited the effect of angiotensin II on the afferent but not the efferent arteriole (Ito et al., 1993; Ito et al., 1991). Another approach utilized was infusion of Ang II into animals in which NOS was inhibited. These studies demonstrate that renal vasoconstriction induced by Ang II is greatly augmented (Baylis et al., 1994; Sigmon et al., 1994). For instance, the cortical blood flow is very sensitive to infusion of Ang II after NOS blockade while the medullary circulation, although sensitive to NOS blockade, is relatively insensitive to Ang II infusion (Madrid et al., 1997; Zou et al., 1998).

In summary, these studies indicate that NO opposes the vasoconstrictive effects of Ang II on the afferent arteriole and the cortical and to a lesser extent medullary blood flow but does not appear to have any significant effect on the efferent arteriole.

*Ang II Regulates NO synthesis.* Recent studies indicate that infusion of Ang II increases NO production. In renal resistance arteries for instance, infusion of Ang II increases NO production (Thorup et al., 1998), an effect that is blocked by Ang II AT<sub>1</sub> receptor blocker (Thorup et al., 1999). Similarly, Ang II infusion into the renal medulla increases medullary NO production (Zou et al., 1998). In the kidney, the effects of Ang II on Na<sup>+</sup> reabsorption and vasoconstriction are mediated by the AT<sub>1</sub> receptor (Hall et al., 1999). Recently, another receptor for Ang II, termed AT<sub>2</sub>, has been identified in the kidney (Ozono et al., 1997). Studies have demonstrated that activation of AT<sub>2</sub> receptors by Ang II results in bradykinin and NO synthesis (Siragy and Carey, 1997). Mice with targeted disruption of the AT<sub>2</sub> receptor have low bradykinin and NO production and demonstrate slightly elevated blood pressure (Hein et al., 1995; Ichiki et al., 1995). Infusion of Ang II greatly increases Na<sup>+</sup> reabsorption and the arterial blood pressure in these mice (Siragy et al., 1999). These studies strongly suggest that AT<sub>2</sub> receptors counteract the effects of AT<sub>1</sub> receptors on vascular tone and Na<sup>+</sup> reabsorption by inducing bradykinin and NO production.

The mechanism of Ang II-mediated NO synthesis regulation is unclear. However, acute and chronic administration of Ang II upregulates eNOS expression in endothelial cells (Hennington et al., 1998). On the contrary, Ang II inhibits iNOS expression in a PT-like cell line (Wolf et al., 1997) and in vascular smooth muscle cells (Nakayama et al., 1994). The effect of Ang II on nNOS expression has not been investigated.

In summary, activation of AT<sub>2</sub> receptors by Ang II results in NO synthesis in the kidney and inhibition of both Ang II-induced vasoconstriction and Na<sup>+</sup> reabsorption. Ang II stimulates expression of eNOS and inhibits that of iNOS, whereas its effects on nNOS are unknown. The mechanism of Ang II-mediated NO synthesis in the kidney is therefore unclear but may be dependent on cell-type specific expression of NOS isoforms.

## 1.7 Rationale and Objectives

The general objective of our study was to determine the role of NO and nNOS in the regulation of Na<sup>+</sup> excretion by the kidney.

1) NO is produced in the kidney and is an important regulator of renal blood flow and a potent natriuretic factor. The natriuresis induced by NO is due to both a hemodynamic effect and a direct inhibitory effect of NO on tubular Na<sup>+</sup> reabsorption. The proximal tubule reabsorbs 65% of filtered Na<sup>+</sup>, mostly due to the activity of the NHE3 transporter expressed on the apical membrane. Therefore, the objectives of our first study were:

- 1) To determine whether NO regulates NHE3-mediated Na<sup>+</sup> uptake in the PT.
- 2) To determine the signaling pathway involved in NO-mediated NHE3 regulation.

2) During ingestion of a high dietary salt the production of NO increases and facilitates excess Na<sup>+</sup> excretion. NO is an important mediator of pressure natriuresis, since blockade of medullary nNOS activity during high salt intake causes Na<sup>+</sup> retention and development of hypertension. The localization inner medullary nNOS remains unknown. Since iNOS has been reported to be expressed in the renal tubules, it

may also be involved in the regulation of pressure natriuresis. Since the mechanism by which dietary salt increases renal NO synthesis is unknown the objectives of our second study were:

1) To determine whether nNOS is expressed in IMCD.

2) To determine whether high dietary salt regulates expression of mRNA and protein for nNOS and iNOS in the kidney.

3) Chronic renal failure is a state of low renal NO production. Administration of L-arginine to rats with CRF increases GFR and prevents proteinuria and development of glomerulosclerosis, suggesting that decreased renal NO production may accelerate renal impairment. Similarly, treatment with captopril, an inhibitor of Ang II synthesis, prevents progression of renal failure. The mechanisms mediating inhibition of renal NO production are unknown but may be due to downregulation of NOS isoform expression.

Therefore, the objectives of our third study were:

1) To determine the effect of 5/6 Nx on cortical and inner medullary nNOS expression.

2) To determine whether Ang II AT<sub>1</sub> receptors regulate renal nNOS expression.

4) CRF is associated with low renal NO production. This may be in part due to decreased expression of NOS isoforms. Indeed in the previous study we have shown that nNOS mRNA and protein are significantly decreased in the cortex and medulla of 5/6 Nx rats. Furthermore, increased circulating levels of an endogenous NOS inhibitor, ADMA, have been detected in patients with CRF. An endogenous inhibitor of nNOS, termed PIN, has been identified in the brain but its expression in the kidney is unknown.

Therefore, the objectives of our fourth study were:

1) To determine whether PIN is expressed in the kidney.

2) To determine whether PIN expression is altered in 5/6 Nx rats.

**CHAPTER 2****Nitric Oxide Stimulates Guanylate Cyclase and Regulates Sodium Transport in  
Rabbit Proximal Tubule**

Agnes Roczniak and Kevin D. Burns

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A. Roczniak and K. D. Burns wrote the manuscript. A. Roczniak performed all the experiments with the exception of PKA activity assays which were performed by K. D. Burns.

Please note that a portion of the results that were used to generate Figure 2.3 were included in my 4<sup>th</sup> year honors thesis in the Department of Physiology.

**ABSTRACT**

The proximal tubule contains the target for nitric oxide (NO), soluble guanylate cyclase, and has the capacity for NO production. Inhibition of renal NO synthesis reduces fractional excretion of lithium, suggesting an inhibitory effect of NO on proximal tubule Na<sup>+</sup> transport. The present studies determined direct effects of donors of NO in rabbit proximal tubule. In both freshly isolated proximal tubule segments and in primary cultures of proximal tubule cells, sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) caused dose-dependent increases in cyclic guanosine monophosphate (cGMP). SNAP was more potent than SNP in stimulating cGMP: this was associated with an enhanced production of nitrite, the stable end-product of NO. In rabbit proximal tubule cells, SNP or SNAP (10<sup>-3</sup> M) significantly inhibited the activity of the apical Na<sup>+</sup>-H<sup>+</sup> exchanger, determined by assay of amiloride-sensitive <sup>22</sup>Na<sup>+</sup> uptake (% inhibition: SNP: 34.90 ± 5.52%; p < 0.001; SNAP: 30.77 ± 8.20%; p < 0.002). To determine the role of cGMP in mediating these effects, proximal tubule cells were incubated with the membrane-permeable analogue, 8-Br-cGMP. Na<sup>+</sup>-H<sup>+</sup> exchange was significantly inhibited by 8-Br-cGMP (10<sup>-3</sup> M) (% inhibition: 32.40 ± 9.06%; p < 0.05). The inhibitor of soluble guanylate cyclase, LY-83583, caused partial inhibition of SNP-stimulated cGMP generation, and partly blocked the inhibitory effect of SNP on Na<sup>+</sup>-H<sup>+</sup> exchange. Protein kinase A (PKA) activity was not stimulated by SNP, indicating that potential cross-activation of PKA by cGMP did not mediate the effects of NO-donors. These data indicate that NO stimulates soluble guanylate cyclase in rabbit proximal tubule, and causes inhibition of Na<sup>+</sup>-H<sup>+</sup> exchange. This is at least partly mediated by generation of cGMP. We conclude that NO is an important autocrine or paracrine factor directly regulating Na<sup>+</sup> transport in the proximal tubule.

## INTRODUCTION

Nitric oxide (NO) exerts potent effects on renal blood flow, glomerular hemodynamics, and urinary Na<sup>+</sup> excretion (Alberola et al., 1992; De Nicola et al., 1992; Lahera et al., 1993; Lahera et al., 1991; Majid et al., 1993). In dogs, inhibition of endogenous NO synthesis increases renal vascular resistance, and reduces renal blood flow, urine flow, and natriuresis (Majid et al., 1993; Nakamura et al., 1993). Inhibition of NO synthesis also augments renal fractional lithium clearance, suggesting that NO might inhibit proximal tubule Na<sup>+</sup> reabsorption (Alberola et al., 1992; Nakamura et al., 1993). In contrast, recent *in vivo* studies in rat demonstrated that inhibition of NO production reduced proximal tubule solute transport, and augmented the inhibitory effect of high concentrations of angiotensin II on proximal solute reabsorption (De Nicola et al., 1992).

At least some of these diverse effects of NO are postulated to be secondary to the well-described vasodilatory actions of NO: possible direct effects of NO on tubular epithelial cell function are incompletely understood. In this regard, in mouse cortical collecting duct cells, NO was shown to inhibit Na<sup>+</sup> reabsorption, possibly via generation of cyclic guanosine monophosphate (cGMP) (Stoos et al., 1992). In microdissected rat cortical collecting ducts, NO caused inhibition of H<sup>+</sup>-ATPase activity (Tojo et al., 1994), suggesting a role for NO in H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> transport.

NO production is catalyzed by nitric oxide synthase (NOS), of which multiple isoforms have been cloned (Schmidt et al., 1993). Interestingly, the proximal tubule tonically expresses mRNA for the inducible form of NOS (NOS-II), determined by *in situ* hybridization (Ahn et al., 1994), and reverse-transcription polymerase chain reaction (RT-PCR) studies (Mohaupt et al., 1994). In both rat (Markewitz et al., 1993) and human (McLay et al., 1994) proximal tubule cells in culture, exposure to cytokines stimulates production of NO. In addition, mRNA for the constitutive form of NOS (NOS-I) has been detected in several nephron segments (Terada et al., 1992), and a recent study reported the presence of the endothelial nitric oxide synthase (NOS-III) mRNA in rat proximal tubule (Ujiiie et al., 1994). Thus, locally-generated NO could possibly serve as an autocrine or paracrine factor to renal tubular epithelial cells.

The present study was performed to determine the direct effects of exogenous donors of NO in rabbit proximal tubule cells, with respect to signaling mechanisms, and effects on apical Na<sup>+</sup>-H<sup>+</sup> exchange. The

results indicate that NO may act as an important autocrine or paracrine regulator of Na<sup>+</sup> reabsorption in the proximal tubule.

## MATERIALS AND METHODS

### Cell Culture

Primary cultures of rabbit proximal tubule cells were prepared as previously described (Burns et al., 1993), by a modification of the method of Vinay et al. (Vinay et al., 1981). Briefly, renal cortices from New Zealand white female rabbits (2.5-3.5 kg) were gently minced and suspended in a solution containing (in mM) 115 NaCl, 24 NaHCO<sub>3</sub>, 5 KCl, 1.5 CaCl<sub>2</sub>, 1.0 MgSO<sub>4</sub>, 2.0 NaH<sub>2</sub>PO<sub>4</sub>, 5 glucose, 1 alanine, 10 *N*-2-hydroxyethylpiperazine-*N'*,2-ethanesulfonic acid (HEPES; pH 7.4), 0.03% collagenase (Sigma type I, St. Louis, MO), and 0.01% soy-bean trypsin inhibitor (Sigma) (buffer A), gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> and maintained at 37°C for 30 min. Following collagenase digestion, the cortical suspension was strained through a 250 µM brass sieve and centrifuged for 1 min at 100 x g. The pellet was resuspended in buffer A without collagenase or trypsin inhibitor and washed and recentrifuged 3 times at 100 x g for 1 min each. The pellet was then mixed with a 50% Percoll solution of identical ionic composition as buffer A, and which had been previously chilled to 4°C. The Percoll solution was centrifuged at 26,000 x g for 30 min at 4°C. Following centrifugation, the tissue separated into 4 distinct bands, as described by Vinay et al. (Vinay et al., 1981). The lowermost band (F4), enriched in proximal tubule segments, was removed, washed, and plated onto 35-mm plastic culture dishes. In some experiments, the F4 layer was washed and resuspended in DME/F12 with 0.1% bovine serum albumin (BSA: Sigma Fraction V), supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), for assays of cyclic adenosine monophosphate (cAMP) or cGMP. Cells plated for culture were initially grown in a defined medium of DME/F12, insulin (2.5 µg/ml), transferrin (2.5 µg/ml), selenium (5 nM), hydrocortisone (50 nM) and 3,3',5-triiodo-*L*-thyronine (2.5 nM), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Twenty four hours after plating, cells were switched to defined medium with 0.5% fetal bovine serum. Cultures were incubated in a humidified atmosphere of 5% CO<sub>2</sub>/95% room air, at 37°C, and were fed every 2-3 days. Cells were used for experiments at confluency, usually reached 5-7 days after plating.

### **Assay of cGMP**

For measurement of cGMP levels in freshly isolated proximal tubule suspensions, the F4 layer was preincubated for 15 min at 37°C in DME/F12 with 0.1% BSA, supplemented with 0.5 mM IBMX. Agonists were then added for a further 5 min incubation, and the reaction was stopped by addition of ice-cold 10% trichloroacetic acid (TCA; v/v, final concentration). Suspensions were kept on ice for 30 min, and then spun at 4°C in a Hermle Z-320K microcentrifuge at 12,000 rpm for 10 min. Aliquots of supernatant were extracted 4 times with 4 volumes of water-saturated ether, brought to pH 7.0 with tris(hydroxymethyl)aminomethane (Tris), and assayed for cGMP, by radioimmunoassay using a commercially available kit (cGMP kit, Amersham, Oakville, Ont.). For assay of cGMP in primary cultures of rabbit proximal tubule cells, confluent monolayers were incubated for 15 min at room temperature in DME/F12 supplemented with 0.5 mM IBMX. Agonists were then added for a further 5 min at room temperature, and the reaction was stopped by aspiration of the media and addition of ice-cold 10% TCA. Cell-associated cGMP content was determined as described above. Results are expressed as pmoles cGMP/mg protein.

### **Assay of cAMP**

Levels of cAMP in response to arginine vasopressin or parathyroid hormone were determined in fresh suspensions of rabbit proximal tubules, and in primary cultures. Conditions were as described for cGMP assay in tubule suspensions, except that exposure to agonists was for 10 min. cAMP levels were determined by a radioligand binding assay, using a commercial kit (cAMP kit, Amersham).

### **Determination of nitrite (NO<sub>2</sub><sup>-</sup>) levels**

The levels of NO<sub>2</sub><sup>-</sup> were measured in primary cultures of rabbit proximal tubule cells, utilizing the Griess reaction, essentially as described (Green et al., 1982). Briefly, the media from confluent cells grown on 35-mm dishes was aspirated, and cells were incubated for 20 min in the presence or absence of exogenous donors of NO, in fresh, defined medium supplemented with 0.5% fetal bovine serum, at room temperature. Aliquots of the incubation medium (0.5 ml) were then removed and added to 1 ml of Griess reagent (1:1 (v/v); 1% sulfanilamide in 30% acetic acid and 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in 60% acetic acid). The NO<sub>2</sub><sup>-</sup> reacts with the Griess reagent to form a chromophore (Green et al., 1982), and its absorbance

at 550 nm was measured after 10 min in a Beckman DU-7 spectrophotometer. The concentrations of nitrites were determined from a standard curve, established by adding known quantities of sodium nitrite to the media used for the assay.

### **Assay of Na<sup>+</sup>-H<sup>+</sup> exchange**

The activity of the apical Na<sup>+</sup>-H<sup>+</sup> exchanger was determined in primary cultures of rabbit proximal tubule cells, by measurement of 5-(*N*-ethyl-*N*-isopropyl)-amiloride- (EIPA; Research Biochemicals Int., Natick, MA) or amiloride-sensitive <sup>22</sup>Na<sup>+</sup> uptake, as previously described (Burns et al., 1991). In preliminary experiments, it was determined that cytosolic acidification with nigericin/KCl-containing solutions of pH 5.8-6.4 caused inhibition of exogenous nitrate-stimulated cGMP production. Thus, all subsequent experiments on Na<sup>+</sup>-H<sup>+</sup> exchange were performed on non-acidified cells. Briefly, confluent cell monolayers were preincubated for 60 min at 37°C in a Na<sup>+</sup>-free solution containing (in mM): 137 choline chloride, 5 KCl, 5 glucose, 1.0 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, brought to pH 7.3 with Tris (buffer B). Cells were then incubated further for 15 min in buffer B, with or without agonists, at room temperature. This solution was then aspirated, and the cells were incubated in a solution identical to buffer B, except that it contained 134 mM choline chloride, 3 mM NaCl, 1 μCi/ml <sup>22</sup>Na<sup>+</sup> (sp. act. 1437.37 mCi/mg, DuPont, Mississauga, Ont.), and 0.5 mM ouabain, with or without 50 μM EIPA or 1 mM amiloride (Burns et al., 1991). In some experiments, cells were preincubated with the inhibitor of soluble guanylate cyclase, 6-anilinoquinoline-5,8-quinone (LY-83583; Calbiochem, San Diego, CA), prior to measurement of <sup>22</sup>Na<sup>+</sup> uptake. Preliminary experiments determined that amiloride-sensitive uptake of <sup>22</sup>Na<sup>+</sup> was linear for up to 15 min, and substitution of EIPA for amiloride yielded similar results. After 5 min, the <sup>22</sup>Na<sup>+</sup>-containing solution was aspirated, and the cells were washed 4 times with ice-cold 100 mM MgCl<sub>2</sub>. Cells were solubilized in 1 N NaOH, and incorporated radioactivity determined by liquid scintillation counting. All assays were performed in duplicate. Na<sup>+</sup>-H<sup>+</sup> exchange activity was determined as the difference between <sup>22</sup>Na<sup>+</sup> uptake in the absence and in the presence of amiloride or EIPA, and was calculated as nmoles/mg protein/5 min.

### Assay of Na<sup>+</sup>-phosphate cotransport

The activity of Na<sup>+</sup>-phosphate cotransport in proximal tubule cells was determined as the Na<sup>+</sup>-dependent uptake of <sup>32</sup>P (1 mCi/ml; Amersham). Cells were preincubated for 15 min at room temperature in Na<sup>+</sup>-free solution (buffer B), in the presence or absence of agonists. This solution was then aspirated and the uptake of <sup>32</sup>P (1.5 μCi/ml) proceeded over 5 min at room temperature, either in buffer B, or in a solution identical to buffer B but containing 127 mM choline chloride and 10 mM NaCl, a concentration of NaCl well below the  $K_{mNa^+}$  for Na<sup>+</sup>-phosphate cotransport (Gmaj and Murer, 1986). After 5 min, <sup>32</sup>P-containing solutions were aspirated, and the cells were washed 4 times with ice-cold phosphate-buffered saline. Cells were solubilized in 1 N NaOH, and cell-associated radioactivity measured by liquid scintillation counting.

### Assay of protein kinase A (PKA) in permeabilized cells

PKA activity was assayed in rabbit proximal tubule cells, utilizing a permeabilized cell assay, as previously utilized by us (Burns and Harris, 1995). Cells were grown to confluence on 96-well dishes, and preincubated at 23°C in a solution of (in mM) 140 choline chloride, 5 KCl, 1.8 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 5 glucose, 10 HEPES (pH 7.3), with or without agonists, for 15 min. To initiate the assay, the preincubation solution was aspirated and replaced with 100 μl of permeabilization solution (130 mM KCl, 10 mM HEPES (pH 7.3), 10 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 25 mM β-glycerophosphate, 100 μM (g-<sup>32</sup>P) ATP (~5000 cpm/pmol), 100 μg/ml digitonin, 0.5 mM IBMX, and 100 μM PKA peptide substrate, Kemptide (Peninsula Laboratories, Inc., Belmont, CA) and the reaction allowed to proceed at 23°C for 15 min. Agonists were not present in the permeabilization solution: the assay determines kinase activity stimulated by agonists prior to cell permeabilization. The assay was terminated by addition of 11 μl of 100% TCA. Aliquots (60 μl) of the reaction mixture were then spotted onto 2 x 2 cm phosphocellulose papers (Whatman P-81) and the papers were washed 4 times with 1% phosphoric acid. Bound phosphopeptides were quantitated by scintillation spectrometry. Background phosphorylation, determined in the absence of the specific PKA peptide substrate, was subtracted from each measurement to determine peptide-specific phosphorylation. Preliminary experiments revealed that the rate of phosphorylation was linear for at least 20 min.

**Protein measurement**

Proteins were quantified by Lowry assay (21), using BSA as standard.

**Statistics**

Results are expressed as means  $\pm$  SEM. For single comparisons, the Student's t test was utilized. For multiple group comparisons, ANOVA was used, with the Bonferroni correction. A value of  $p < 0.05$  was considered significant.

## RESULTS

### Characterization of proximal tubule suspensions and cells

Direct effects of NO on cortical collecting duct have been reported (Stoos et al., 1992; Tojo et al., 1994). Thus, it was essential that our proximal tubule preparation be highly enriched. By colorimetric assay (Rutenburg, 1966), ~95% of cells in the F4 layer of the Percoll gradient stained positively for alkaline phosphatase, an enzyme marker for proximal tubule (n= 4). In addition, assays of cAMP were performed on freshly isolated rabbit proximal tubule segments. Parathyroid hormone ( $10^{-7}$  M) significantly stimulated cAMP production ( $7.65 \pm 2.54$ -fold increase;  $p < 0.05$ ; n= 9), whereas arginine vasopressin ( $10^{-7}$  M) had no effect ( $1.03 \pm 0.11$ -fold of control;  $p = \text{ns}$ ; n= 10). These results suggested that the F4 suspensions were enriched in proximal tubules, with negligible contamination with cortical collecting ducts. Furthermore, in fresh suspensions of rabbit proximal tubules, atrial natriuretic peptide (ANP:  $10^{-7}$  M) significantly increased cGMP production (cGMP: control:  $0.54 \pm 0.12$  vs ANP:  $1.31 \pm 0.20$  pmol/mg protein;  $p < 0.05$ ; n= 3), in agreement with the recent study of Eitle et al. (Eitle et al., 1994).

At confluence, primary cultures of proximal tubule cells were also characterized with respect to presence of alkaline phosphatase. Virtually all cells stained strongly positive for the presence of this enzyme, on three separate cell preparations. In addition, scanning electron microscopy revealed the presence of apical brush border surface, characteristic of proximal tubule cells, in greater than 99% of cultured cells. Finally, in primary cultures, cAMP responses to hormones remained characteristic of proximal tubule (PTH ( $10^{-7}$  M):  $2.17 \pm 0.24$ -fold increase;  $p < 0.005$ ; n= 3; AVP ( $10^{-7}$  M):  $1.09 \pm 0.11$ -fold of control;  $p = \text{ns}$ ; n= 3).

### Effect of donors of NO on cGMP in proximal tubule

NO stimulates cGMP production in many cell types, via activation of soluble guanylate cyclase (Schmidt et al., 1993). Experiments were performed to determine if exogenous NO-donors stimulated cGMP production in proximal tubule. In freshly isolated suspensions of proximal tubules, both sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) caused concentration-dependent increases in cGMP production (Figure 2.1). Considerable variability in cGMP responses was observed from rabbit to rabbit. Levels of cGMP peaked at  $10^{-4}$  M of SNP (control:  $1.31 \pm 0.31$  vs SNP:  $6.54 \pm 1.79$  pmol/mg protein;  $p <$

0.005; n= 12-15) or  $10^{-5}$  M SNAP (control:  $0.90 \pm 0.16$  vs SNAP:  $7.25 \pm 1.42$  pmol/mg protein;  $p < 0.001$ ; n= 8). Greater than 90% of tubular cells remained viable following incubation times required for the cGMP assay, as determined by exclusion of trypan blue.

To determine if this response was maintained in primary cultures of rabbit proximal tubule cells, confluent cells were incubated with varying concentrations of SNP or SNAP. As shown in Figure 2.2, both compounds caused stimulation of cGMP production. Compared to freshly isolated tubules, cGMP responses in cells did not appear to reach maximum at  $10^{-3}$  M of exogenous NO donors. Cyclic GMP assays in primary cultures were performed at 23°C, since all subsequent transport studies were performed at this temperature. However, in primary cultures, incubation at 37°C was not associated with significantly increased cGMP levels in response to donors of NO ( $10^{-6}$ - $10^{-3}$  M), compared to responses at 23°C (data not shown).

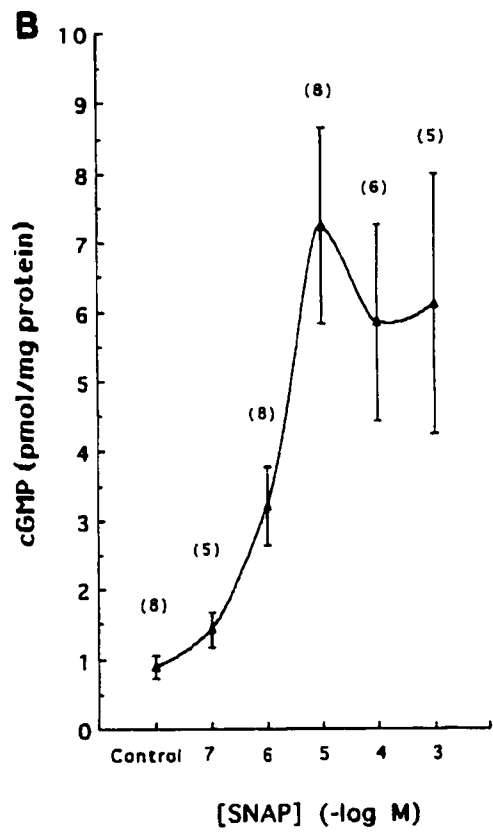
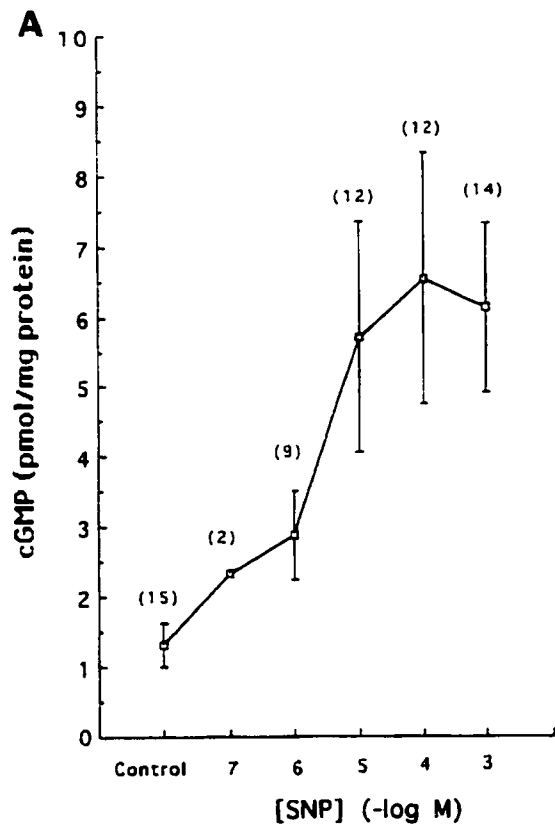
In primary cultures of rabbit proximal tubule cells, significant stimulation of cGMP occurred at a concentration of  $10^{-3}$  M SNP (control:  $0.49 \pm 0.07$  vs SNP:  $1.84 \pm 0.29$  pmol/mg protein;  $p < 0.001$ ; n= 17-19). SNAP significantly stimulated cGMP levels at concentrations of  $10^{-4}$  M or greater (control:  $0.49 \pm 0.13$  vs SNAP ( $10^{-4}$  M):  $1.52 \pm 0.10$  pmol/mg protein;  $p < 0.025$ ; n= 3-4). These results suggested that soluble guanylate cyclase activity is present in proximal tubule, and that cells in primary culture maintain expression of this phenotype.

### **NO<sub>2</sub><sup>-</sup> levels**

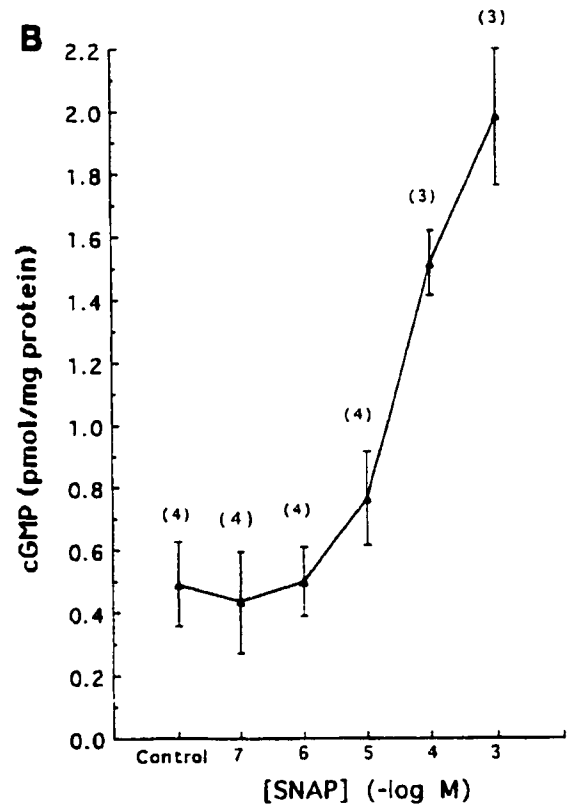
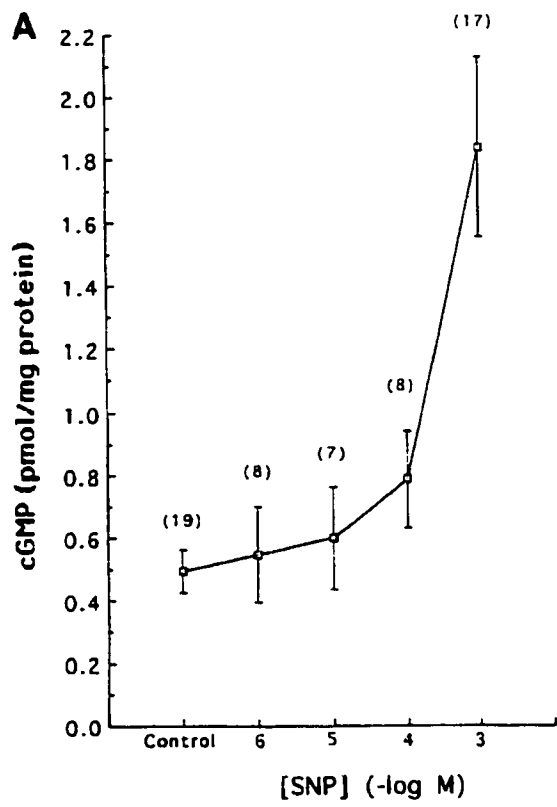
The concentration of NO in solution can be estimated by determination of the levels of NO<sub>2</sub><sup>-</sup>, a stable metabolite of NO (Marletta et al., 1988). Since SNAP stimulated cGMP production at lower concentrations than SNP, NO<sub>2</sub><sup>-</sup> levels were measured in rabbit proximal tubule cells, following exposure to SNP or SNAP for the same duration as in cGMP assays (Table 2.1). Incubation of cells with either SNP or SNAP caused concentration-dependent increases in NO<sub>2</sub><sup>-</sup> production, with significantly greater production observed with SNAP.

**Figure 2.1: SNP and SNAP stimulate cGMP production in rabbit proximal tubular segments.**

Freshly isolated proximal tubular segments were preincubated for 15 min in the presence of IBMX (0.5 mM), and then exposed to the NO-donors, SNP (Figure 2.1A) or SNAP (Figure 2.1B), for 5 min at 37°C. The reaction was stopped by addition of 10% TCA, and the levels of cGMP determined by radioimmunoassay. Results are means  $\pm$  SEM of experiments performed in duplicate. Values in parentheses represent numbers of individual experiments.



**Figure 2.2: SNP and SNAP stimulate cGMP production in primary cultures of rabbit proximal tubule cells.** Primary cultures of rabbit proximal tubule cells were preincubated for 15 min in the presence of IBMX (0.5 mM), and then exposed to SNP (Figure 2.2A) or SNAP (Figure 2.2B) for 5 min at 24°C. The reaction was stopped with 10% TCA, and the levels of cGMP determined by radioimmunoassay. Results are means  $\pm$  SEM, of experiments performed in duplicate. Values in parentheses are numbers of experiments.



**Table 2.1. Effect of SNP or SNAP on nitrite production in rabbit proximal tubule cells**

<b>Condition</b>	<b>Nitrite level (nmol/mg protein/20 min)</b>	<b>n</b>
<b>Control</b>	<b>0.094 ± 0.022</b>	<b>6</b>
<b>SNP (10<sup>-6</sup> M)</b>	<b>0.103 ± 0.022</b>	<b>3</b>
<b>SNP (10<sup>-5</sup> M)</b>	<b>0.179 ± 0.021</b>	<b>4</b>
<b>SNP (10<sup>-4</sup> M)</b>	<b>0.321 ± 0.033</b>	<b>4</b>
<b>SNP (10<sup>-3</sup> M)</b>	<b>0.613 ± 0.080</b>	<b>4</b>
<b>SNAP (10<sup>-4</sup> M)</b>	<b>0.852 ± 0.117*</b>	<b>4</b>
<b>SNAP (10<sup>-3</sup> M)</b>	<b>3.840 ± 0.300†</b>	<b>4</b>

Values are means ± SEM of experiments performed in duplicate. Numbers in parentheses represent number of individual experiments. \* p < 0.05 vs SNP (10<sup>-4</sup> M); † p < 0.002 vs SNP (10<sup>-3</sup> M).

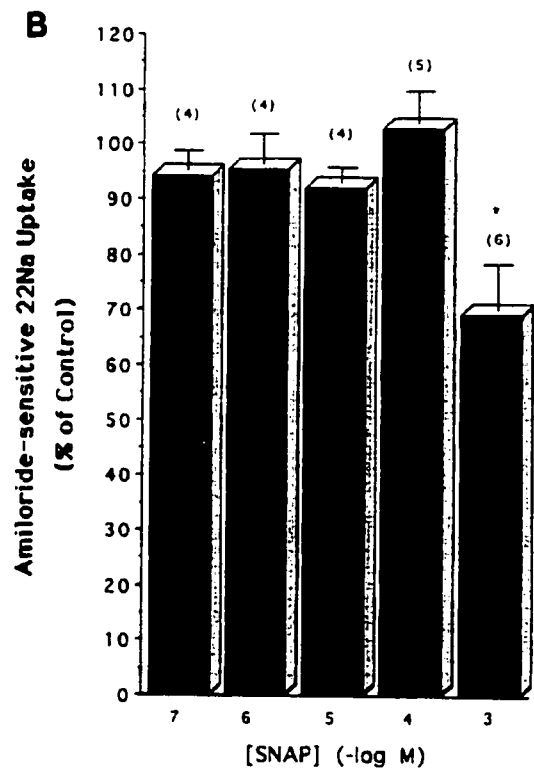
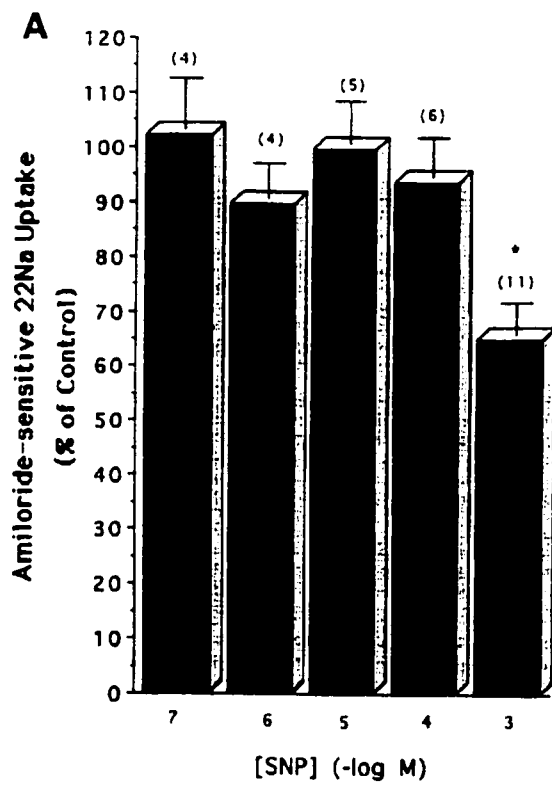
### Effect of donors of NO on Na<sup>+</sup>-H<sup>+</sup> exchange

NO directly inhibits Na<sup>+</sup> transport in the cortical collecting duct (Stoos et al., 1992), and has been suggested to directly affect proximal Na<sup>+</sup> transport (Alberola et al., 1992; De Nicola et al., 1992; Guzman et al., 1995; Nakamura et al., 1993). To determine if NO regulates Na<sup>+</sup> transport in proximal tubule, EIPA- or amiloride-sensitive <sup>22</sup>Na<sup>+</sup> uptake was measured in non-acidified cells preincubated in Na<sup>+</sup>-free solution. Preliminary experiments confirmed that the Na<sup>+</sup>-H<sup>+</sup> exchanger in these cells was relatively amiloride-resistant, with no significant activity observed at concentrations of EIPA greater than 50 μM, and no significant inhibition at concentrations less than 1 μM (not shown). This suggests that the cells were exclusively expressing the apical membrane, amiloride-resistant form of the Na<sup>+</sup>-H<sup>+</sup> exchanger.

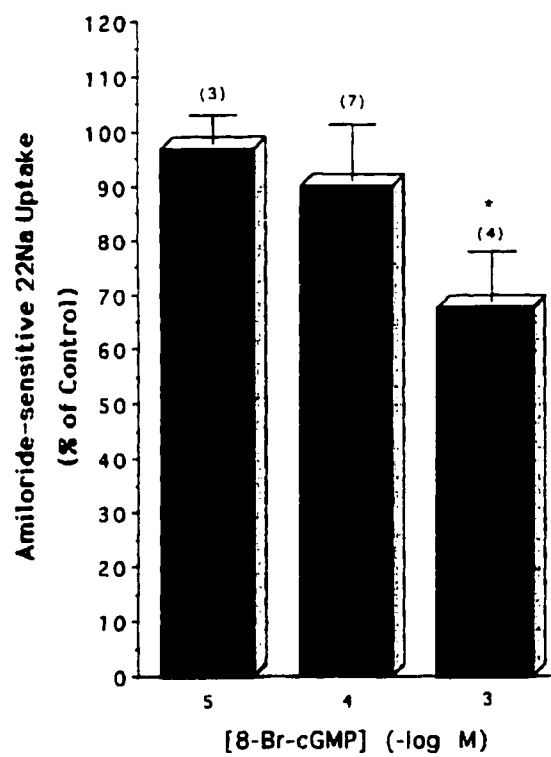
In non-acidified cells, preincubation with SNP (10<sup>-3</sup> M) for 15 min caused a significant inhibition of amiloride- or EIPA-sensitive <sup>22</sup>Na<sup>+</sup> uptake (control: 0.72 ± 0.11 vs SNP: 0.45 ± 0.07 nmol/mg protein (% inhibition: 34.90 ± 5.52%); p < 0.001; n = 11) (Figure 2.3A). Lower concentrations of SNP, from 10<sup>-7</sup>-10<sup>-4</sup> M, had no significant effect on Na<sup>+</sup>-H<sup>+</sup> exchange. SNP (10<sup>-3</sup> M) induced a small but significant increase in EIPA-insensitive <sup>22</sup>Na<sup>+</sup> uptake (control: 0.55 ± 0.04 vs SNP: 0.70 ± 0.06 nmol/mg protein; p < 0.05; n = 14). In separate experiments, the addition of 2 mM NaCl to buffered solutions, to control for the presence of Na<sup>+</sup> in SNP (SNP contains 2 Na<sup>+</sup> for each nitroprusside moiety), had no significant effect on Na<sup>+</sup>-H<sup>+</sup> exchange activity under the conditions of our assay (EIPA-sensitive <sup>22</sup>Na uptake: 105.55 ± 12.76% of control; p = ns; n = 4).

Guanylate cyclase activity may be inhibited at decreased pH (Wong and Garbers, 1992). Since cell Na<sup>+</sup> depletion is associated with lowering of pH<sub>i</sub>, it is possible that responses to exogenous NO donors could be modified by this maneuver. The cGMP responses to SNP (10<sup>-3</sup> M) were compared in cells incubated in isotonic solution containing 140 mM NaCl, vs Na<sup>+</sup>-depleted cells, preincubated in choline chloride-containing buffer. No significant difference in cGMP generation was observed (NaCl 140 mM: 3.53 ± 0.36-fold increase in cGMP; Na<sup>+</sup>-depletion: 2.94 ± 0.54-fold increase; p = ns; n = 4).

**Figure 2.3: SNP and SNAP inhibit Na<sup>+</sup>-H<sup>+</sup> exchange in rabbit proximal tubule cells.** Cells were preincubated for 60 min in Na<sup>+</sup>-free solution, exposed to SNP (Figure 2.3A) or SNAP (Figure 2.3B) for 15 min, and then assayed for EIPA-sensitive <sup>22</sup>Na<sup>+</sup> uptake. Results are expressed as % of control value. Values in parentheses are numbers of experiments performed in duplicate. \* p < 0.001 vs control (Figure 2.3A), or \* p < 0.002 vs control (Figure 2.3B).



**Figure 4: Effect of 8-BrcGMP on Na<sup>+</sup>-H<sup>+</sup> exchange in rabbit proximal tubule cells.** Cells were preincubated for 60 min in Na<sup>+</sup>-free solution, exposed to 8-BrcGMP for 15 min, and then assayed for EIPA-sensitive <sup>22</sup>Na<sup>+</sup> uptake. Values in parentheses represent numbers of experiments performed in duplicate. \*p < 0.05 vs control.



To determine whether the inhibitory effect on  $\text{Na}^+\text{-H}^+$  exchange was specific for SNP, cells were preincubated with varying concentrations of the NO-donor SNAP, prior to assay of  $^{22}\text{Na}^+$  uptake. As shown in Figure 2.3B, SNAP ( $10^{-3}$  M) also caused significant inhibition of apical  $\text{Na}^+\text{-H}^+$  exchange ( $69.33 \pm 8.20\%$  of control;  $p < 0.002$ ;  $n=6$ ). Furthermore, these concentrations of SNP or SNAP did not affect cell viability during the assay, as determined by trypan blue exclusion.

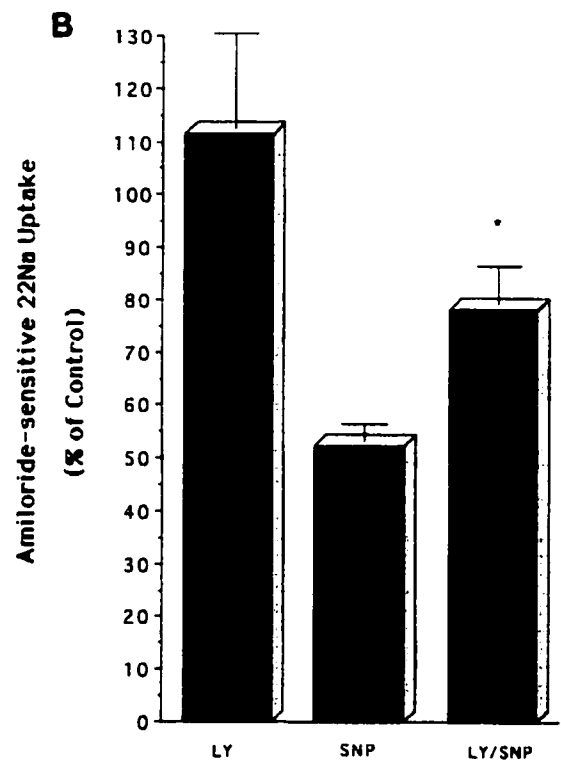
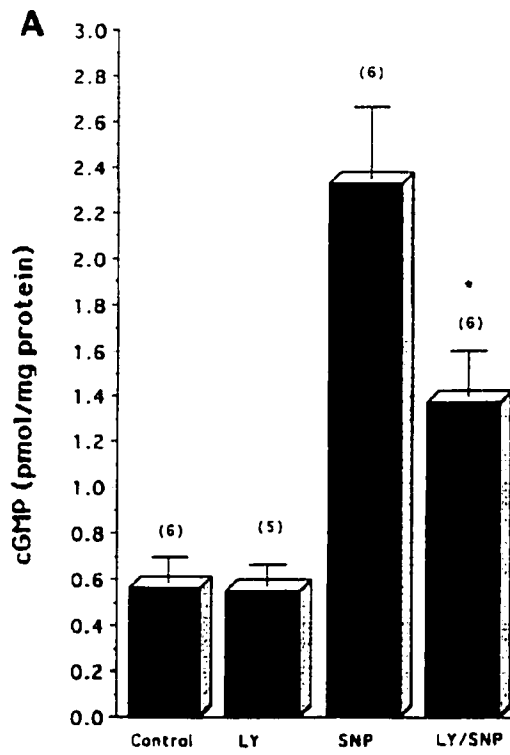
### **Role of cGMP**

Cyclic GMP has been shown to inhibit  $\text{Na}^+\text{-H}^+$  exchange in other cell types (Caramelo et al., 1994; Semrad et al., 1990). However, the role of this second messenger in regulation of proximal tubule apical  $\text{Na}^+\text{-H}^+$  exchange is incompletely understood. The results with the donors of NO suggested that cGMP might at least partially mediate the inhibitory effects on proximal tubule  $\text{Na}^+\text{-H}^+$  exchange. Experiments were performed to determine the effects of the membrane-permeable analogue of cGMP, 8-BrcGMP, on apical  $\text{Na}^+\text{-H}^+$  exchange. Preincubation of non-acidified cells for 15 min with 8-BrcGMP ( $10^{-3}$  M) caused a  $32.40 \pm 9.06\%$  inhibition of  $\text{Na}^+\text{-H}^+$  exchange ( $p < 0.05$ ;  $n=4$ ). No significant effect was observed at either  $10^{-5}$  M or  $10^{-4}$  M (Figure 2.4).

To investigate further the role of cGMP in regulation of the  $\text{Na}^+\text{-H}^+$  exchanger, cells were preincubated with the inhibitor of soluble guanylate cyclase, LY-83583 ( $10^{-4}$  M), prior to exposure to SNP ( $10^{-3}$  M) and assay of  $\text{Na}^+\text{-H}^+$  exchange. LY-83583 had no significant effect on basal levels of cGMP, but significantly blunted the increase in cGMP induced by SNP (cGMP: SNP:  $2.33 \pm 0.31$  vs SNP/LY-83583:  $1.38 \pm 0.20$  pmol/mg protein;  $p < 0.02$ ;  $n=6$ ) (Figure 2.5A). In addition, in the presence of LY-83583, SNP ( $10^{-3}$  M) did not significantly inhibit  $\text{Na}^+\text{-H}^+$  exchange (% inhibition: SNP alone:  $47.60 \pm 2.98\%$ ;  $p < 0.02$  vs control; SNP/LY-83583:  $21.80 \pm 7.37\%$ ;  $p = \text{ns}$  vs control) (Figure 2.5B). However, the difference between the inhibitory effect of SNP alone and the effect of SNP/LY-83583 did not achieve statistical significance, by ANOVA testing. These results suggested that activation of guanylate cyclase at least partially mediates NO-induced inhibition of  $\text{Na}^+\text{-H}^+$  exchange.

**Figure 2.5: A) Effect of guanylate cyclase inhibitor, LY-83583, on SNP-stimulated cGMP production in rabbit proximal tubule cells.** Cells were preincubated for 30 min with or without LY-83583 (LY:  $10^{-4}$  M), and then incubated with or without SNP ( $10^{-3}$  M) for 5 min, followed by measurement of cGMP levels by radioimmunoassay. Results are means  $\pm$  SEM of experiments performed in duplicate, with values in parentheses representing numbers of individual experiments. \*  $p < 0.02$  vs SNP.

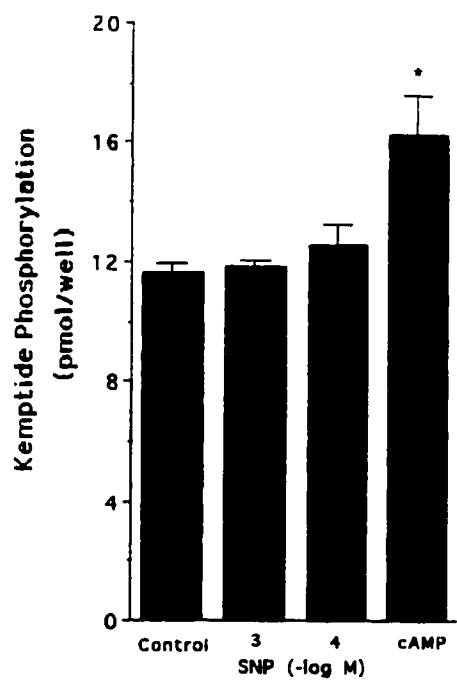
**B) Effect of LY-83583 on SNP-induced inhibition of  $\text{Na}^+$ - $\text{H}^+$  exchange.** Cells were preincubated for 30 min in the presence or absence of LY-83583 (LY:  $10^{-4}$  M) in  $\text{Na}^+$ -free solution, and then incubated for 15 min with or without SNP ( $10^{-3}$  M), prior to assay of EIPA-sensitive  $^{22}\text{Na}^+$  uptake. Results are means  $\pm$  SEM of 5 experiments performed in duplicate. \*  $p = \text{ns}$  vs LY or control.



**Role of cross-activation of PKA by cGMP**

High concentrations of cGMP have been shown to cross-activate PKA (Cornwell et al., 1994; Forte et al., 1992; Schmidt et al., 1993). Experiments were performed to determine if cross-activation of PKA was involved in the response of proximal tubule cells to incubation with NO-donors, since, for example, cAMP has been shown to inhibit apical  $\text{Na}^+\text{-H}^+$  exchange (Burns et al., 1991), perhaps by a PKA-dependent mechanism. Primary cultures of rabbit proximal tubule cells were preincubated with or without SNP for 15 min, and then permeabilized with digitonin, followed by assay of phosphorylation of a specific peptide substrate for PKA (Kemptide). Incubation of cells with cAMP ( $2.7 \times 10^{-9}$  M) caused a significant stimulation of Kemptide phosphorylation (control:  $11.62 \pm 0.31$  vs cAMP:  $16.26 \pm 1.36$  pmol/well;  $p < 0.05$ ;  $n = 3-4$ ) (Figure 2.6). Incubation with SNP did not stimulate phosphorylation (SNP ( $10^{-3}$  M):  $11.84 \pm 0.20$ ; SNP ( $10^{-4}$  M):  $12.58 \pm 0.69$  pmol/well;  $p = \text{ns}$  vs control;  $n = 3$ ).

**Figure 2.6: SNP does not induce cross-activation of PKA in rabbit proximal tubule cells.** Cells were preincubated for 15 min with vehicle (control), SNP, or cAMP ( $2.7 \times 10^{-9}$  M), prior to permeabilization and assay of PKA, as described in "Materials and Methods". Results are means  $\pm$  SEM of 3-4 experiments in duplicate. \*  $p < 0.05$  vs control.



**Effect of NO on Na<sup>+</sup>-phosphate cotransport**

As noted above, in the present studies, SNP significantly stimulated EIPA-insensitive <sup>22</sup>Na<sup>+</sup> uptake in proximal tubule cells. To determine if this might be due to effects on Na<sup>+</sup>-phosphate cotransport, experiments were performed to measure the effects of SNP on the Na<sup>+</sup>-dependent uptake of <sup>32</sup>P in these cells. SNP (10<sup>-3</sup> M) caused a marked stimulation of cotransport activity (SNP: 156.50 ± 13.11% of control; p < 0.002; n = 6). This increase in cotransport activity also occurred when cells were incubated in the presence of EIPA (50 μM), indicating that it was not due to inhibition of Na<sup>+</sup>-H<sup>+</sup> exchange and resultant increased transcellular gradient for Na<sup>+</sup> entry (data not shown). Furthermore, when cells were incubated in the presence of an additional 2 mM NaCl as a control for the effects of incubation with 10<sup>-3</sup> M SNP, no significant effect on <sup>32</sup>P uptake occurred under the conditions of the assay (% increase: 16.90 ± 7.47%; p = ns; n = 3).

To determine if the stimulatory effect of SNP on Na<sup>+</sup>-phosphate cotransport might be mediated by generation of cGMP, cells were preincubated with 8-Br-cGMP (10<sup>-3</sup> M), prior to assay of <sup>32</sup>P uptake. 8-Br-cGMP caused a small but significant stimulation of cotransport activity (% stimulation: 15.25 ± 2.50%; p < 0.05; n = 4). In contrast to these observations, incubation of cells with SNAP did not significantly affect Na<sup>+</sup>-phosphate cotransport (SNAP 10<sup>-4</sup> M: 120.25 ± 15.31% of control; p = ns; n = 4; SNAP 10<sup>-3</sup> M: 96.67 ± 7.97% of control; p = ns; n = 3).

## DISCUSSION

The present study demonstrates that donors of NO exert direct effects in rabbit proximal tubule. First, we have shown that NO stimulates proximal tubule soluble guanylate cyclase, leading to generation of cGMP. Second, donors of NO inhibit apical Na<sup>+</sup>-H<sup>+</sup> exchange. Third, studies utilizing the permeable analogue of cGMP, 8-Br-cGMP, and the inhibitor of guanylate cyclase, LY-83583, provide evidence that direct effects of NO donors on proximal tubule transport are at least partly mediated by the cGMP pathway.

### **Evidence for the proximal tubule as a target for NO**

The potent effects of NO as a vasodilator and neurotransmitter are well characterized. However, the role of NO in directly regulating tubular epithelial cell function is poorly understood. In this regard, the importance of the proximal tubule as a potential target for direct action of NO is borne out by several observations. Terada et al. recently reported that the rat proximal tubule contained the highest levels along the nephron of mRNA for the target of NO, soluble guanylate cyclase (Terada et al., 1992). This suggests that NO, synthesized in renal endothelial cells, may diffuse to the proximal tubule and stimulate cGMP production. In addition, the presence of both inducible NOS and endothelial NOS mRNA in proximal tubule (Ahn et al., 1994; Mohaupt et al., 1994; Ujiie et al., 1994) suggests that NO may act as a paracrine or autocrine factor in this segment. This is supported by studies in the proximal tubule-like cell line, LLC-PK<sub>1</sub>, in which basal synthesis of NO has been observed, associated with enhanced cGMP production (Ishii et al., 1991).

Our data suggest a role for either renal endothelial- or tubular epithelial cell-derived NO in stimulating soluble guanylate cyclase in proximal tubule. Concentration-dependent effects of SNP or SNAP on cGMP generation were observed in both fresh proximal tubule segments and primary cell cultures. The relatively low cGMP response of cultured cells to NO donors was not due to conduction of experiments at room temperature, but may reflect partial loss of guanylate cyclase activity in primary culture. Characterization studies did not reveal contamination of the proximal tubule preparation with endothelial cells or other tubular segments, indicating that NO-donors were acting directly on proximal tubule. Furthermore, our data is in agreement with the preliminary study of Lortie et al. (Lortie et al., 1994), in which SNP stimulated cGMP production in freshly isolated proximal tubule cells.

Of the two NO-donors utilized, SNAP was more potent in stimulating guanylate cyclase. This is likely secondary to increased production of NO by SNAP with these assay conditions, as suggested by nitrite measurements (Table I). Increased potency of SNAP compared to SNP in cGMP generation has also been observed in studies in brain tissue slices (Southam and Garthwaite, 1991). Furthermore, although nitrite levels may not represent a precise estimate of NO concentration (Schmidt et al., 1993), it must be noted that the levels achieved with SNP or SNAP in the present study are comparable to, if not less than, those in studies on cytokine-induced NO release in cultured proximal tubule cells (Markewitz et al., 1993; McLay et al., 1994). Thus, our data support the possibility that endogenous renal NO might act on proximal tubule cells.

### **Effects of NO on proximal tubule transport**

Pharmacological inhibition of intrarenal NO synthesis has been shown to reduce urine  $\text{Na}^+$  excretion, and urine flow, in the absence of changes in glomerular filtration rate (Lahera et al., 1991; Majid et al., 1993). The present study provides evidence for direct effects of NO donors on proximal tubule  $\text{Na}^+$  reabsorption. Significant inhibitory effects of SNP or SNAP on EIPA-sensitive apical  $^{22}\text{Na}$  uptake were observed. The relative EIPA-resistance of this uptake confirmed that it represented the proximal tubule  $\text{Na}^+-\text{H}^+$  exchanger. Although relatively high concentrations of NO-donors were required for inhibition, these concentrations have been utilized in recent studies in glomeruli (Craven et al., 1994), cortical collecting duct (Tojo et al., 1994), smooth muscle (Cornwell et al., 1994), and transfected Chinese hamster ovary cells (Stadler et al., 1994). In intact tissue, stimulation of guanylate cyclase requires concentrations of NO donors orders of magnitude higher than those needed in broken cell preparations (Southam and Garthwaite, 1991), suggesting that rapid inactivation of NO occurs in intact tissue.

In the present study, experiments were performed on basal  $\text{Na}^+-\text{H}^+$  exchange activity in non-acidified cells, since we determined that cytoplasmic acidification inhibited NO-donor-induced cGMP generation. The cause of this is unclear, although addition of weak acid dissociates the heme group from guanylate cyclase, associated with loss of response to SNP and other NO donors (Wong and Garbers, 1992). Although we did not measure  $\text{pH}_i$  in this study,  $\text{Na}^+$ -depletion was unlikely to have profoundly decreased  $\text{pH}_i$ , since cGMP responses were similar in  $\text{Na}^+$ -depleted cells compared to cells in 140 mM NaCl. In our previous study on LLC-PK<sub>1</sub> cells,  $\text{Na}^+$  depletion was associated with a  $\text{pH}_i$  of  $7.15 \pm 0.04$  (Burns et al., 1991). Since apical  $\text{Na}^+$ -

H<sup>+</sup> exchange mediates most of proximal tubule Na<sup>+</sup> transport, our data suggest a mechanism whereby endogenous NO could induce natriuresis *in vivo*. Furthermore, NO has recently been shown to inhibit Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in mouse proximal tubule cells (Guzman et al., 1995), and to inhibit Na<sup>+</sup> transport in cortical collecting duct cells (Stoos et al., 1992), suggesting a coordinated inhibitory effect on tubular Na<sup>+</sup> reabsorption.

Hammond et al. have shown that ANP inhibits renal phosphate transport, perhaps via cGMP production (Hammond et al., 1985). In contrast, a recent study by Khraibi demonstrated that inhibition of intrarenal NO synthesis caused inhibition of proximal tubule phosphate reabsorption, suggesting that NO stimulates phosphate transport (Khraibi, 1994). In the present study, a stimulatory effect of SNP on proximal tubule cell Na<sup>+</sup>-phosphate cotransport was observed. This may have accounted for the SNP-induced increase in EIPA-insensitive <sup>22</sup>Na<sup>+</sup> uptake in these cells. However, although 8-Br-cGMP caused a small but significant stimulation of Na<sup>+</sup>-phosphate cotransport, SNAP did not induce the same effect. Thus, our data do not permit us to conclude that NO stimulates proximal tubule Na<sup>+</sup>-phosphate cotransport, although it is possible that SNAP may exert nonspecific inhibitory effects on cotransporter activity at high concentrations. The possibility of stimulatory effects of NO on other amiloride-insensitive Na<sup>+</sup>-dependent transporters, such as Na<sup>+</sup>-glucose or Na<sup>+</sup>-amino acid cotransport, must also be considered.

### **Role of cGMP**

In the present study, incubation of proximal tubule cells with 8-Br-cGMP significantly inhibited Na<sup>+</sup>-H<sup>+</sup> exchange. In addition, LY-83583 partially inhibited SNP-stimulated cGMP production, and partially blocked the inhibitory effect on Na<sup>+</sup>-H<sup>+</sup> exchange. Of note, inhibition of Na<sup>+</sup>-H<sup>+</sup> exchange was not observed at NO-donor concentrations less than 10<sup>-3</sup> M, perhaps due to insufficient stimulation of cGMP production. This is supported by our data with use of the NO-donor SNAP (10<sup>-4</sup> M), which stimulated cGMP production in proximal tubule cells to a level not significantly different from that observed with use of SNP (10<sup>-3</sup> M)/LY-83583, and which did not inhibit Na<sup>+</sup>-H<sup>+</sup> exchange at this concentration (Figure 3B). Taken together, the results strongly suggest that NO donors inhibit proximal tubule Na<sup>+</sup>-H<sup>+</sup> exchange at least partly via a cGMP-dependent pathway. In cultures of vascular smooth muscle cells (Caramelo et al., 1994), and in avian intestinal cells (Semrad et al., 1990), both ANP and cGMP inhibit Na<sup>+</sup>-H<sup>+</sup> exchange activity, possibly via

activation of cGMP-dependent protein kinase. Furthermore, our data are in agreement with recent *in vivo* rat studies by Lahera et al. (Lahera et al., 1993), in which infusion of 8-BrcGMP prevented the reduction in renal excretory function induced by NO synthesis inhibition.

#### **Role of cGMP-independent activity of NO**

Although a major action of NO-donors is on the cGMP signaling pathway, high concentrations of NO can interact with iron-, heme-, and thiol-containing cellular proteins, leading to alterations in enzymatic function (Rutenburg, 1966). In addition, high concentrations of cGMP cross-activate cAMP-dependent protein kinase in both smooth muscle cells (Cornwell et al., 1994) and in intestine (Forte et al., 1992). In the present study, no effect of SNP on phosphorylation of a cAMP-dependent protein kinase peptide substrate was observed. Thus, the data do not support a role for NO-induced cross-activation of PKA and resultant inhibition of apical  $\text{Na}^+$ - $\text{H}^+$  exchange by this pathway. However, the possible involvement of other cGMP-independent pathways cannot be ruled out from this study.

In summary, we have demonstrated direct effects of NO-donors on rabbit proximal tubule cGMP generation and transport function. Inhibition of proximal tubule  $\text{Na}^+$ - $\text{H}^+$  exchange by NO, mediated at least in part by cGMP, may play a role in induction of natriuresis *in vivo*. The presence of NOS and guanylate cyclase in the proximal tubule suggest that NO may be an important paracrine or autocrine regulator of  $\text{Na}^+$  transport.

## CHAPTER 3

### **Effect of Dietary Salt on Neuronal Nitric Oxide Synthase in the Inner Medullary Collecting Duct**

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A. Rocznik and K. D. Burns wrote the manuscript. A. Rocznik performed all the experiments. J. Zimpelmann established the procedure for IMCD isolation in the laboratory and microdissected IMCD tubules for immunofluorescence.

**ABSTRACT**

Nitric oxide (NO) derived from neuronal NO synthase (nNOS) in the kidney inner medulla has been implicated in the regulation of arterial blood pressure. The purpose of the present study was to determine the effect of high dietary NaCl on the expression of nNOS in the rat inner medullary collecting duct (IMCD). After 3 days or 3 weeks of high (4.0%) NaCl diet in rats, urinary  $\text{NO}_2^-/\text{NO}_3^-$  excretion significantly increased. In freshly microdissected IMCD, nNOS was readily detected by immunofluorescence with polyclonal antibody, an effect that was completely blocked by neutralization of antibody with immunizing antigen. In rats fed a 4.0% NaCl diet for 3 days, IMCD nNOS mRNA, detected by RT-PCR, did not change from control values (0.3% NaCl:  $19.84 \pm 1.57 \times 10^3$  vs. 4.0% NaCl:  $20.44 \pm 3.14 \times 10^3$  cpm,  $P = \text{ns}$ ,  $n = 3$ ). By Western blotting however, nNOS protein expression significantly increased (0.3% NaCl:  $0.51 \pm 0.12$  vs 4.0% NaCl:  $0.92 \pm 0.14$  arbitrary units,  $P < 0.05$ ,  $n = 5$ ). After 3 weeks of 4.0% dietary NaCl, expression of nNOS mRNA and protein in IMCD did not differ significantly from control values. In contrast to these data, renal cortical expression of nNOS mRNA and protein were significantly decreased after 4.0% NaCl diet for 3 days. High dietary NaCl had no significant effect on expression of mRNA for inducible NO synthase (iNOS) in IMCD after either 3 days or 3 weeks. In summary, our data indicate that nNOS mRNA and protein are expressed in IMCD and that high dietary NaCl differentially regulates nNOS expression in IMCD and cortex. The early increase in nNOS protein in IMCD may contribute to enhanced local production of NO, and thereby represent an adaptive response to salt intake.

## INTRODUCTION

Nitric oxide (NO) regulates glomerular hemodynamics, renal blood flow and induces natriuresis (Kone and Baylis, 1997). Within the kidney, the capacity to synthesize NO has been demonstrated in both tubular epithelium and vascular endothelium. Recently, the renal medulla has been shown to produce the highest levels of NO in the kidney (Zou and Cowley, 1997). In this regard, there are at least three isoforms of nitric oxide synthase (NOS) that could contribute to medullary NO production: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (ecNOS). In the medulla, abundant nNOS mRNA has been demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) in inner medullary collecting ducts (IMCD), with lesser expression in the vasa recta and the inner medullary thin limb (Terada et al., 1992). Bachmann et al., however, demonstrated immunoreactivity for nNOS in the macula densa and afferent arteriole, but did not detect nNOS in IMCD (Bachmann et al., 1995). With regards to iNOS mRNA, studies have demonstrated its presence in IMCD and inner medullary interstitial cells (Ahn et al., 1994). Histochemical analysis of renal tissue, however, has not detected basal iNOS protein expression in the IMCD (Aiello et al., 1997). In contrast, ecNOS mRNA and protein are distributed in endothelial cells throughout the renal vasculature, while expression of this isoform in renal tubules is not consistently detected (Singh et al., 1996; Ujiie et al., 1994).

In rats, chronic infusion of an inhibitor of NOS directly into the renal medullary interstitium results in a selective decrease in medullary blood flow, associated with salt and water retention and development of hypertension (Mattson et al., 1994). Inhibition of nNOS expression by intramedullary antisense oligonucleotide administration increases blood pressure in rats maintained on a high salt diet, suggesting that the ability of the kidneys to excrete sodium may be impaired in the absence of medullary nNOS activity (Mattson and Bellehumeur, 1996). Furthermore, during high dietary salt intake, the serum concentration and urinary excretion of the NO decomposition products  $\text{NO}_2^-$  and  $\text{NO}_3^-$  increase (Shultz and Tolins, 1993), suggesting that enhanced NO production represents an adaptive mechanism that induces vasodilatation and natriuresis, thereby contributing to the maintenance of normal blood pressure.

The potential importance of nNOS-derived NO in the inner medulla led us to examine whether this isoform is expressed in tubular cells of the rat IMCD, and to test the hypothesis that high dietary NaCl increases nNOS expression in this segment. Our results demonstrate that nNOS protein is readily detected in

IMCD by immunofluorescence, and that high dietary NaCl differentially regulates nNOS mRNA and protein expression in the IMCD and renal cortex. Whereas high dietary NaCl inhibits nNOS mRNA and protein expression in the cortex, it increases nNOS protein expression without affecting mRNA levels in the IMCD. In contrast, high dietary NaCl has no effect on iNOS mRNA expression in the IMCD.

## **MATERIALS AND METHODS**

### **Animals**

Male Sprague-Dawley rats (200-250 gm) were fed a standard (0.3% NaCl, control) or high (4.0% NaCl) salt diet (Harlan Teklad, Madison, WI) for either 3 days or 3 weeks, with unlimited access to water. The diets were equivalent in contents of all other electrolytes. For 24 hrs prior to sacrifice, the rats were placed in metabolic cages for urine collection. After sacrifice, the kidneys were immediately removed for dissection of cortices and for isolation of IMCD. Kidneys from two rats were pooled for each IMCD preparation.

### **IMCD preparation**

Rat IMCDs were isolated using a modification of the method of Grenier et al. (Grenier, 1978), essentially as described (Kohan et al., 1992). Briefly, renal papillary tissue was rapidly minced and suspended in Krebs buffer (in mM): 118 NaCl, 14 glucose, 25 NaHCO<sub>3</sub>, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1.8 MgSO<sub>4</sub>, and 1.8 KH<sub>2</sub>PO<sub>4</sub> containing 0.1% collagenase A (type I; Boehringer Mannheim, Laval, Que., Canada) and 0.01% deoxyribonuclease (Boehringer Mannheim). The minced tissue was incubated for 120 min at 37°C in 5% CO<sub>2</sub>-95% air with gentle agitation. After collagenase digestion, the tubule suspension was pelleted by centrifugation at 100 x g for 30 sec at room temperature and washed three times with Krebs buffer. The pellet was then reconstituted in a hypotonic solution consisting of Krebs buffer and sterile water (1:2 respectively, vol/vol), gently mixed for 4 min and centrifuged at 100 x g for 30 sec. This procedure has been shown to disrupt all cells except those of the IMCD (Grenier, 1978). The tubules were washed three times with Krebs buffer and then used immediately for either RNA or protein isolation. Cell viability was assessed by Trypan blue exclusion, which demonstrated that at least 95% of IMCD cells were viable.

### **Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from cortex and suspensions of IMCD using a commercially available kit (RNeasy, Qiagen, Chatsworth, CA). Prior to RT-PCR, residual genomic DNA was digested by incubating RNA with amplification grade DNase I (Gibco, Burlington, Ont., Canada) for 15 min at room temperature.

DNase I was inactivated by adding ethylenediaminetetraacetic acid (final concentration, 1.6 mM) followed by incubation at 65°C for 15 min. RNA quality was determined by running samples on 1% agarose-formaldehyde gels stained with ethidium bromide, and RNA concentration and purity were determined by optical density measurement at 260 and 280 nm. RNA samples were uniformly of high quality by these standards.

Serial dilutions of total RNA (6, 30, 150 and 750 ng) were prepared in RNase-free water and reverse-transcribed in a buffer containing 5 mM MgCl<sub>2</sub>, 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 1 mM each of deoxynucleoside triphosphates, 1 μCi γ-(<sup>32</sup>P)- dCTP (specific activity: 3000 Ci/mmmole, Amersham, Mississauga, Ont., Canada), 20 U RNase inhibitor, 50 U Moloney murine leukemia virus reverse transcriptase and 2.5 μM random hexamers (GeneAmp RNA PCR kit, Perkin-Elmer, Norwalk, CT) in a total volume of 20 μl. After reverse transcription, reactions were brought up to 100 μl with 1X PCR buffer supplemented with (final concentrations) 2 mM MgCl<sub>2</sub>, 2.5 U Ampli Taq DNA polymerase (Perkin-Elmer), and 1 μM of either nNOS or iNOS-specific oligonucleotide primers. After initial denaturation at 94°C for 3 min, 33 cycles of amplification (94°C x 30 sec, 68°C (nNOS) or 60°C (iNOS) x 30 sec, 72°C x 30 sec) were performed, followed by final extension at 72°C for 10 min, in a Perkin-Elmer GeneAmp PCR system 2400 apparatus. The PCR products were separated on ethidium bromide-stained 2% agarose gels, the gel bands excised, and the incorporated radioactivity determined by liquid scintillation counting.

To determine relative differences in NOS mRNA abundance in rats on 0.3% and 4.0% NaCl diets, we used the titration analysis method, as described (Michel et al., 1994). This is a noncompetitive, semiquantitative method which utilizes the linear relationship between the logarithm of the initial amount of target mRNA and the logarithm of the amount of amplification product generated, providing that the PCR reactions have not reached plateau phase. To ensure that all data were collected before PCR reactions reached this plateau, we measured <sup>32</sup>P-incorporation into PCR products as a function of cycle number for both nNOS and iNOS mRNA amplifications. In both cases, PCR product amplification was exponential for at least 40 cycles. Accordingly, we used 33 cycles of amplification for all further experiments. To minimize tube-to-tube variation in PCR efficiency we used a master mix of reaction components in all experiments. Under these conditions the logarithm of incorporated radioactivity varied linearly with the logarithm of initial

RNA amount (not shown). To control for variations in RNA isolation and for the efficiency of the reverse transcription reaction,  $\beta$ -actin mRNA was amplified at the time of nNOS and iNOS mRNA amplifications. Total RNA (150 ng) was reverse-transcribed and amplified as described above, in the presence of specific primers for  $\beta$ -actin (20 nM). As a control for any possible genomic DNA contamination, a reaction lacking the reverse transcriptase enzyme was included in all experiments.

The primer sequences for nNOS, iNOS and  $\beta$ -actin products are reported in Table 3.1. The primers for nNOS and iNOS were chosen from the rat brain (Bosse et al., 1995) and murine macrophage (Xie et al., 1992) cDNA sequences, respectively. The specificity of the primers was verified through Gene-Bank.  $\beta$ -actin primers were selected according to the human cDNA sequence (Gunning et al., 1983).

The identity of the nNOS PCR product was confirmed by restriction enzyme digestion with either *Bgl* II or *Sca* I, which generated the expected products (210 and 389 bp, and 101 and 498 bp, respectively). To confirm the fidelity of iNOS RT-PCR, the rat IMCD iNOS PCR product was subcloned, and DNA derived from a single bacterial colony was partially sequenced (92 bp, corresponding to bp 1194-1286 (Xie et al., 1992)) using the Taq Cyclist DNA sequencing kit (Stratagene, La Jolla, CA). This product demonstrated 98% nucleotide identity to the murine macrophage iNOS cDNA (Xie et al., 1992) and 100% nucleotide identity to rat vascular smooth muscle iNOS cDNA (Geng et al., 1994) in the sequenced region.

Table 3.1. PCR primer sequences.

Primers	Sequence	location (Bases)	Reference
<b>nNOS</b>			
Upstream	5'GAATACCAGCCTGA TCCAATGGAACACC3'	2113-2139	(Bredt et al., 1991)
Downstream	5'CTCCAGGAGGGTGT CCACCGCATGCC-3'	2686-2712	
<b>iNOS</b>			
Upstream	5'CACATGCAGAATGA GTACCGG3'	1135-1155	(Xie et al., 1992)
Downstream	5'AGGCTGCCCGGAAG GTTTGTA3'	1819-1840	
<b><math>\beta</math>-Actin</b>			
Upstream	5'ACCGCGAGAAGAT GACCCAGATCATGTT T3'	384-413	(Gunning et al., 1983)
Downstream	5'GCAGCCGTGGCCA TCTCTTGCTCGAAGT C3'	705-734	

## Western blotting

IMCDs from 0.3% and 4.0% NaCl-fed rats were homogenized with a cell disrupter in boiling lysis buffer (1% sodium dodecyl sulphate (SDS), 10 mM Tris-HCl, pH 7.4). The lysate was then boiled for 10 min, followed by centrifugation at 12,000 x g for 2 min to remove insoluble debris. Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad, Montreal, Que., Canada) using bovine serum albumin (BSA, Sigma, St.Louis, MO) as standard. Tubule lysates (20, 40 or 60 µg) were separated on 7.5% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 10% skimmed milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBS-T) and 0.01% sodium azide, for 3 hrs at room temperature. The membranes were then incubated for 18 hrs at 4°C with 1:1000 dilution of mouse monoclonal antibody to nNOS or 1:400 dilution of mouse monoclonal antibody to iNOS (Transduction Laboratories, Lexington, KY). The mouse monoclonal antibody to nNOS was raised against a human peptide fragment of nNOS, and reacts against both human and rat nNOS, at 160 kDa. By Western blot analysis, we determined that this antibody did not cross-react with endothelial cell (ecNOS) protein lysates (Transduction Laboratories), but did detect a 130 kDa band, clearly discernible from nNOS, from cytokine-stimulated macrophages (iNOS), as reported by the manufacturer. In some experiments, two separate rabbit polyclonal antibodies to iNOS were used (Transduction Laboratories, and Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then incubated with 1:2000 dilution of either anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Amersham). Primary antibodies were diluted in TBS-T supplemented with 5% skimmed milk and 0.01% sodium azide, whereas the secondary antibodies were diluted in TBS-T supplemented with 2% milk. Proteins were detected by enhanced chemiluminescence (ECL, Amersham) on Hyperfilm (Amersham), according to the manufacturer's instructions. Prestained standards were used as molecular weight markers (Bio-Rad), and rat pituitary and macrophage lysates (4 µg, Transduction Laboratories) were used as positive controls for nNOS and iNOS, respectively. To control for protein loading, all membranes were stripped and probed with a monoclonal anti-β-actin antibody (mouse ascites fluid, Sigma). Signals on Western blots were quantified by densitometry and corrected for the β-actin signal, using an image analysis software program (NIH Image 1.47).

### **Immunofluorescence studies**

IMCD segments were microdissected from rat kidney medullae, as described (Nadler et al., 1992), dried onto microscope slides for 1 hr at room temperature, and then fixed in a 1:1 (vol/vol) acetone/methanol mixture for 15 min at room temperature. Following fixation, the tubules were permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature. Slides were then incubated in blocking buffer consisting of PBS supplemented with 1% BSA for 30 min. A 1:200 dilution of polyclonal nNOS antibody (Santa Cruz Biotechnology) was added to the slides and incubated for 18 hrs at 4°C. In separate experiments, this antibody did not cross-react with protein lysates (Transduction Laboratories) from cytokine-stimulated macrophages (iNOS) or endothelial cells (ecNOS) on Western blots (not shown), confirming the manufacturer's description regarding its specificity. In contrast, the antibody detected the expected nNOS protein band of 160 kDa in the pituitary and IMCD lysates. IMCDs incubated in the presence of primary antibody which had been previously neutralized with the immunizing antigen were used as negative controls (Santa Cruz Biotechnology). The slides were then washed 3 times for 5 min each in PBS and incubated with donkey anti-rabbit secondary antibody (1:20 dilution, Amersham) conjugated to fluorescein isothiocyanate (FITC), for 60 min at 37°C. All antibodies were diluted in PBS supplemented with 0.3% Triton X-100. Following 3 washes with PBS, the slides were covered with an anti-fade compound (PBS containing 0.1 mM phenylaminediamine and 10% glycerol) and viewed with a Zeiss Axioplan fluorescent microscope. Photographs were taken with Kodak Tri-X black and white film.

### **Urinary nitrate/nitrite content**

Urinary nitrate/nitrite content was determined by the nitrate reductase/Griess reaction assay as described (Shultz and Tolins, 1993). First, nitrate present in the urine samples was reduced to nitrite in the presence of nicotinamide adenine dinucleotide phosphate (150 µg/ml), flavin adenine dinucleotide (3.0 µg/ml) and 0.4 U nitrate reductase (Boehringer Mannheim) for 30 min at room temperature. Nitrite was then measured by combining reduced urine aliquots with a 1:1 mixture (vol/vol) of 1% sulfanilamide (in 30% acetic acid) and 0.1% *N*-(-1-naphthyl)-ethylene diamine dihydrochloride (in 60% acetic acid). Absorbances

were read on an ELISA reader at 550 nm. Nitrate and nitrite content was calculated using nitrate and nitrite standard curves.

### **Urinary sodium excretion**

Urinary sodium concentration was determined by flame photometry (model IL-943, Instrumentation Laboratory, Milano, Italy), kindly performed by the laboratory of Dr. D. Z. Levine (University of Ottawa).

### **Statistics**

Results are presented as means  $\pm$  SEM. Data were analyzed by the unpaired Student T- test. A value of  $p < 0.05$  was considered as significant.

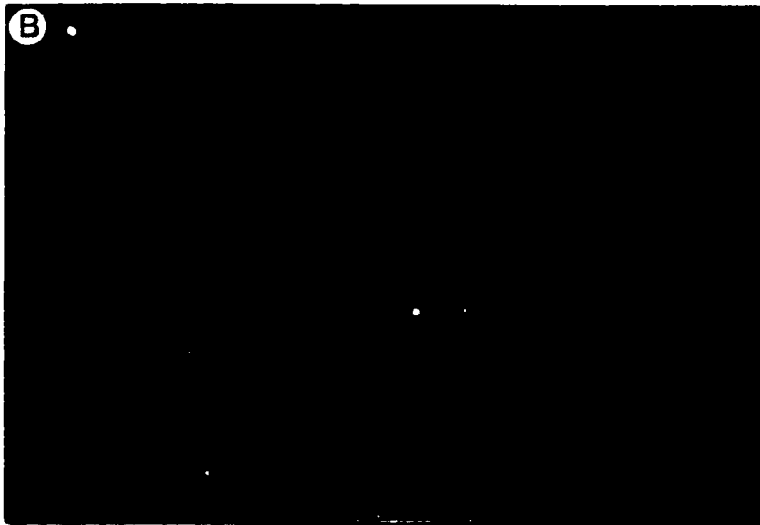
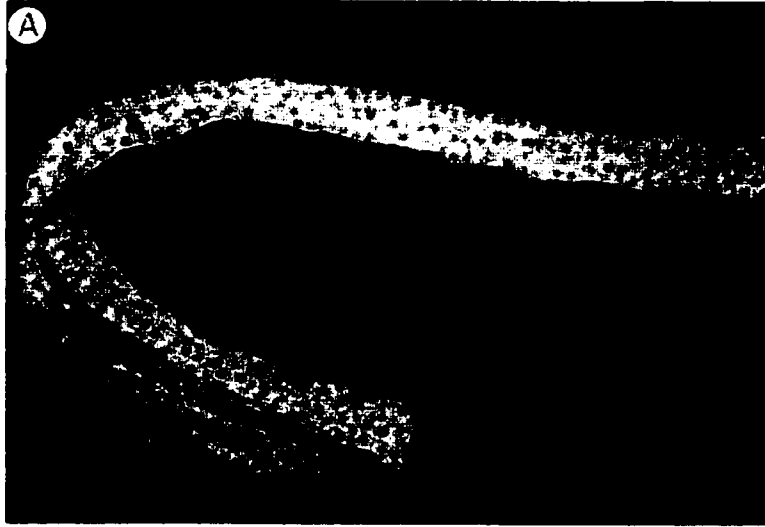
## RESULTS

### **Effect of high dietary NaCl on nNOS mRNA and protein expression in the IMCD and kidney cortex.**

Relatively high levels of nNOS mRNA are found in the IMCD (Terada et al., 1992), yet histochemical methods have been unable to detect nNOS protein in this segment (Bachmann et al., 1995). As shown in Figure 3.1A, using a polyclonal antibody to nNOS, cells within freshly microdissected IMCDs stained strongly for nNOS, with a cytoplasmic pattern. The specificity of the response was verified by staining IMCD with nNOS antibody which had been previously neutralized with immunizing antigen, which resulted in complete loss of immunofluorescence (Figure 3.1B). A monoclonal nNOS antibody (Transduction Laboratories) also strongly stained microdissected IMCD (not shown).

The effect of high dietary NaCl on the expression of nNOS mRNA and protein in IMCD was determined using RT-PCR and Western blotting, respectively. In rats fed a 4.0% NaCl diet for 3 days or 3 weeks, urinary sodium excretion significantly increased (Table 3.2:  $p < 0.001$  vs 0.3% NaCl diet). This was associated with significant increases in urinary  $\text{NO}_2^-/\text{NO}_3^-$  excretion (Figure 3.2, 3 days: 0.3% NaCl:  $2.82 \pm 0.31$  vs 4.0% NaCl:  $3.79 \pm 0.43$   $\mu\text{mol}/100$  g body weight/24 hrs,  $p < 0.05$ ,  $n = 12$ ; 3 weeks: 0.3% NaCl:  $1.21 \pm 0.26$  vs 4.0% NaCl:  $1.92 \pm 0.08$   $\mu\text{mol}/100$  g body weight/24 hrs,  $p < 0.01$ ,  $n = 7$ ). Three days of 4.0% NaCl diet had no effect on nNOS mRNA expression in IMCD (Figure 3.3: 0.3% NaCl:  $19.84 \pm 1.57 \times 10^3$  vs 4.0% NaCl:  $20.44 \pm 3.14 \times 10^3$  cpm,  $p = \text{ns}$ ,  $n = 3$ ) but significantly increased nNOS protein expression in IMCD (Figure 3.4: 0.3% NaCl:  $0.51 \pm 0.12$  vs 4.0% NaCl:  $0.92 \pm 0.14$  arbitrary units,  $p < 0.05$ ,  $n = 5$ ).

**Figure 3.1. Immunolocalization of nNOS in IMCD.** Shown are fluorescence micrographs of microdissected IMCD segments labelled with a polyclonal nNOS antibody (A) or with nNOS polyclonal antibody neutralized by preincubation with immunizing antigen (B). Similar results were obtained in 2 additional experiments.

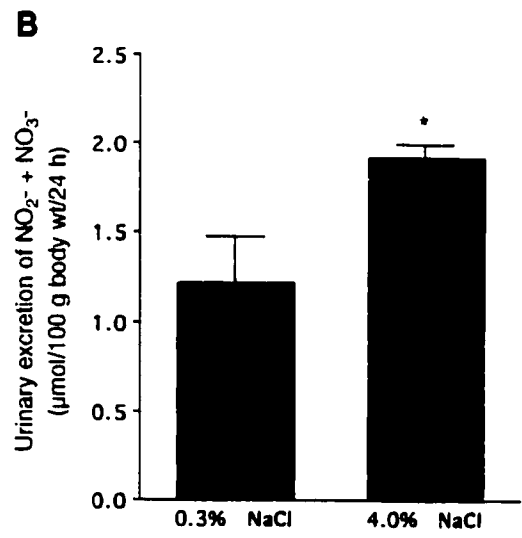
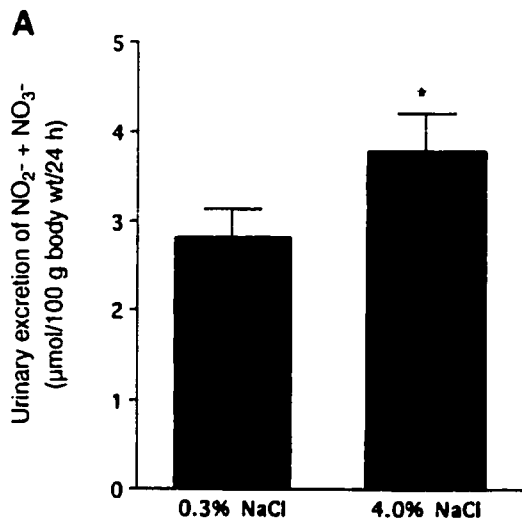


**Table 3.2. Urinary sodium excretion.**

	<b>3 Day, n= 26</b>	<b>3 Week, n= 16</b>
<b>0.3% NaCl diet</b>	0.56 ± 0.06	0.43 ± 0.06
<b>4.0% NaCl diet</b>	10.48 ± 0.50*	10.19 ± 0.48*

Values are means ± SEM in mmol sodium/24h. \*p< 0.001 vs 0.3% NaCl diet.

**Figure 3.2. Effect of high NaCl diet on urinary excretion of  $\text{NO}_2^- + \text{NO}_3^-$ .** Bar graphs depicting 24 hr urinary content of  $\text{NO}_2^- + \text{NO}_3^-$  after 3 days (**A**) and 3 weeks (**B**) of 4.0% NaCl intake. Results are means  $\pm$  SEM (3 day: \*  $P < 0.05$ ,  $n = 12$ ; 3 weeks: \* $p < 0.01$ ,  $n = 7$ ).



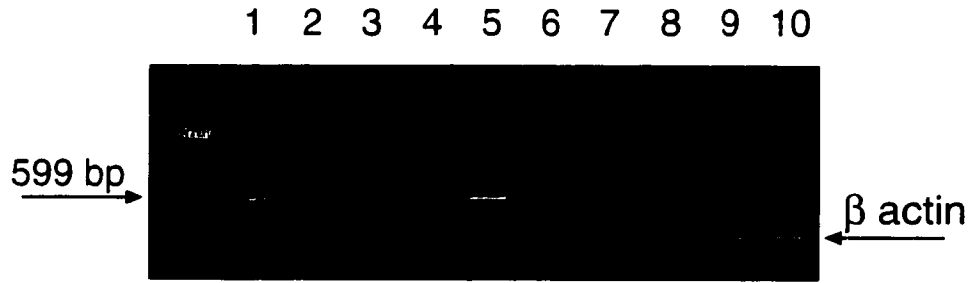
After 3 weeks of 4.0% NaCl intake both nNOS mRNA (Figure 3.5: 0.3% NaCl:  $11.02 \pm 1.05 \times 10^3$  vs 4.0% NaCl:  $13.71 \pm 3.02 \times 10^3$  cpm,  $p = \text{ns}$ ,  $n = 3$ ) and nNOS protein (Figure 3.6: 0.3% NaCl:  $1.03 \pm 0.16$  vs 4.0% NaCl:  $1.13 \pm 0.12$  arbitrary units,  $p = \text{ns}$ ,  $n = 3$ ) in the IMCD did not differ from control values, indicating that the salt-induced nNOS protein upregulation is transient.

Previous studies have demonstrated downregulation of renal cortical nNOS mRNA by high dietary salt (Bredt et al., 1991; Singh et al., 1996). In agreement with these studies, and in contrast to our results in the IMCD, kidney cortical nNOS mRNA significantly decreased after 3 days of 4.0% NaCl intake (Figure 3.7: 0.3% NaCl:  $30.88 \pm 4.36 \times 10^3$  vs 4.0% NaCl:  $16.4 \pm 4.70 \times 10^3$  cpm,  $p < 0.05$ ,  $n = 5$ ). This was associated with a significant decrease in kidney cortical nNOS protein expression, determined by Western blot analysis (Figure 3.8: 0.3% NaCl diet:  $1.88 \pm 0.14$  vs 4.0% NaCl:  $0.92 \pm 0.20$  arbitrary units,  $p < 0.05$ ,  $n = 5$ ).

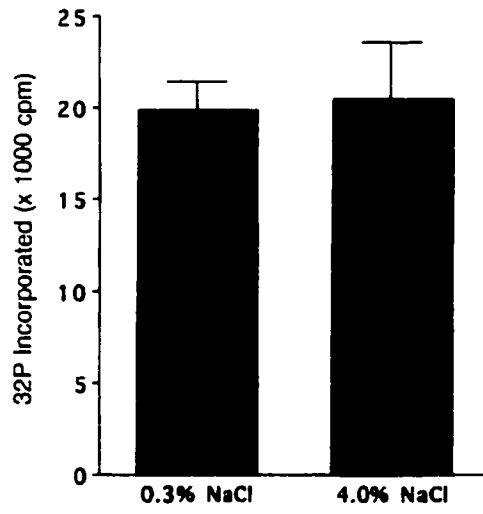
In all experiments, to control for possible variations in RNA isolation and reverse transcription reactions,  $\beta$ -actin mRNA was PCR-amplified along with the nNOS mRNA amplifications. There were no differences in radioactivity incorporated into  $\beta$ -actin PCR products between samples derived from 0.3% or 4.0% NaCl-fed rats (not shown). Similarly, there were no differences in  $\beta$ -actin protein abundance between samples derived from 0.3% or 4.0% NaCl-fed rats, by Western blot analysis.

**Figure 3.3. Effect of 3 day 4.0% NaCl diet on expression of nNOS mRNA in IMCD.** (A) Representative ethidium bromide-stained agarose gel of IMCD nNOS RT-PCR products (599 bp) from 0.3% (lanes 1-4) and 4.0% (lanes 5-8) NaCl-fed rats. Dilutions of total RNA were (from left to right): 750, 150, 30, and 6 ng.  $\beta$ -actin PCR products (350 bp) from IMCD of 0.3% and 4.0% NaCl-fed rats are shown in lanes 9 and 10, respectively. (B) Bar graph depicting radioactivity incorporated into nNOS mRNA amplification products. Results are means  $\pm$  SEM, for 30 ng of initial total RNA (n= 3).

**A**

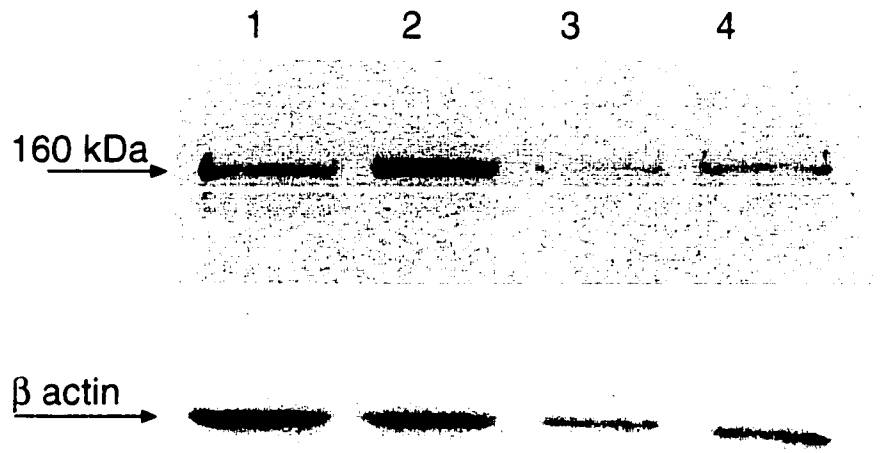


**B**

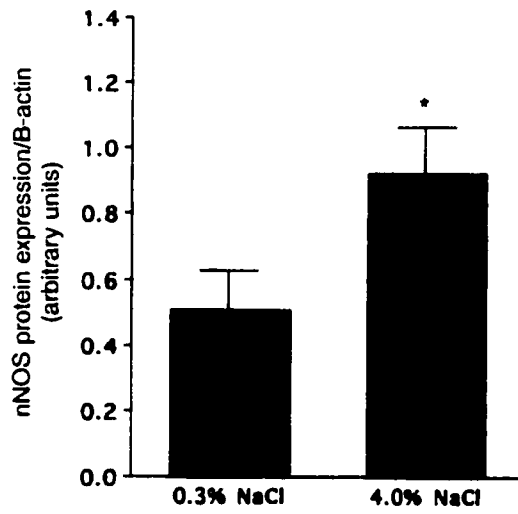


**Figure 3.4. High NaCl diet increases nNOS protein expression in IMCD at 3 days.** (A) Representative Western blot of nNOS protein expression in IMCD of rats fed a 4.0% NaCl diet for 3 days. Lanes 1 and 3: proteins from 0.3% NaCl-loaded rats, 40 and 20  $\mu\text{g}$  of total protein, respectively. Lanes 2 and 4: proteins from 4.0% NaCl-loaded rats, 40 and 20  $\mu\text{g}$  of total protein, respectively. Corresponding bands for  $\beta$ -actin are depicted, demonstrating equal loading. (B) Bar graph depicting densitometric quantification of Western blot signals for nNOS protein IMCD. Results are means  $\pm$  SEM (\*  $p < 0.05$ ,  $n = 5$ ).

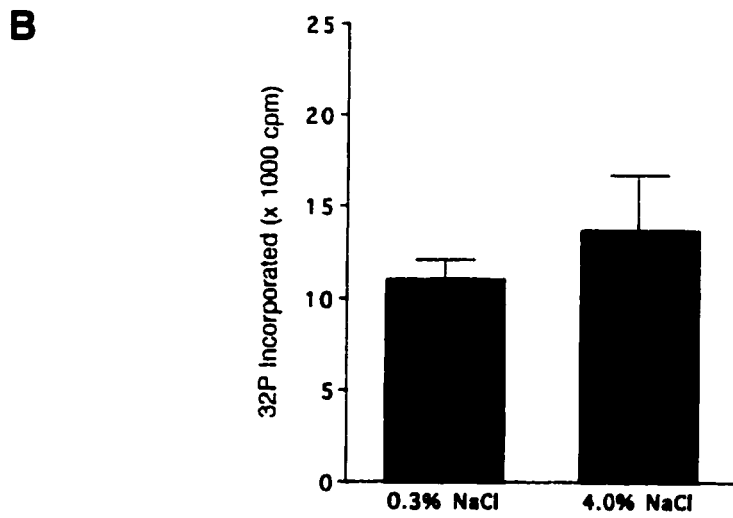
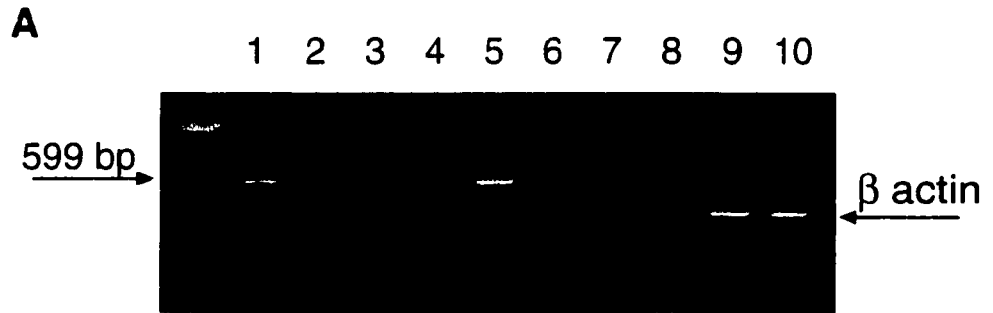
**A**



**B**

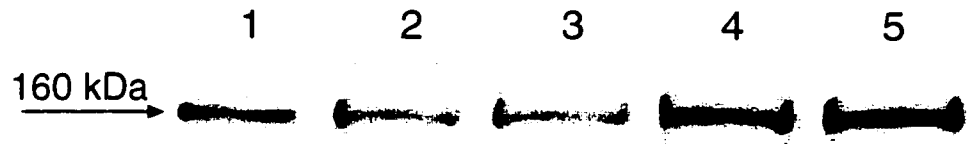


**Figure 3.5. Effect of 3 week 4.0% NaCl diet on expression of nNOS mRNA in IMCD.** (A) Representative ethidium bromide-stained agarose gel of IMCD nNOS RT-PCR products (599 bp) from 0.3% (lanes 1-4) and 4.0% (lanes 5-8) NaCl-fed rats. Dilutions of total RNA were (from left to right): 750, 150, 30, and 6 ng.  $\beta$ -actin PCR products (350 bp) from 0.3% and 4.0% NaCl-fed rats are shown in lanes 9 and 10 respectively. (B) Bar graph depicting radioactivity incorporated into nNOS mRNA amplification products. Results are means  $\pm$  SEM, for 30 ng of initial total RNA (n= 3).

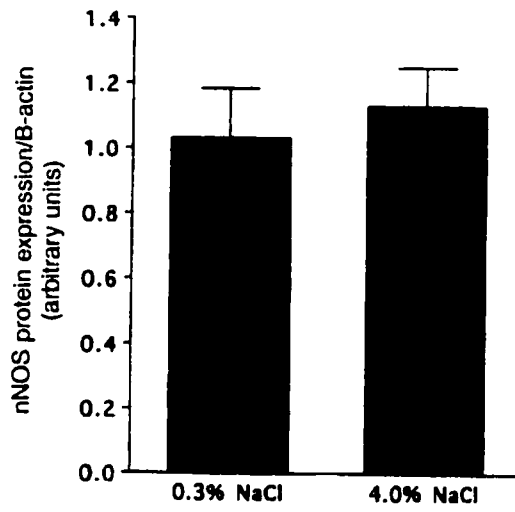


**Figure 3.6. Effect of 3 week 4.0% NaCl diet on nNOS protein expression in IMCD.** (A) Representative Western blot of nNOS protein expression in IMCD of rats fed 4.0% NaCl diet for 3 weeks. Lane 1: rat pituitary lysate (positive control). Lanes 2 and 4: proteins from 0.3% NaCl-fed rats, 20 and 40  $\mu$ g total protein, respectively. Lanes 3 and 5: proteins from 4.0% NaCl-fed rats, 20 and 40  $\mu$ g total protein, respectively. (B) Bar graph depicting densitometric quantification of Western blot signals for nNOS protein in IMCD. Results are means  $\pm$  SEM (n= 3).

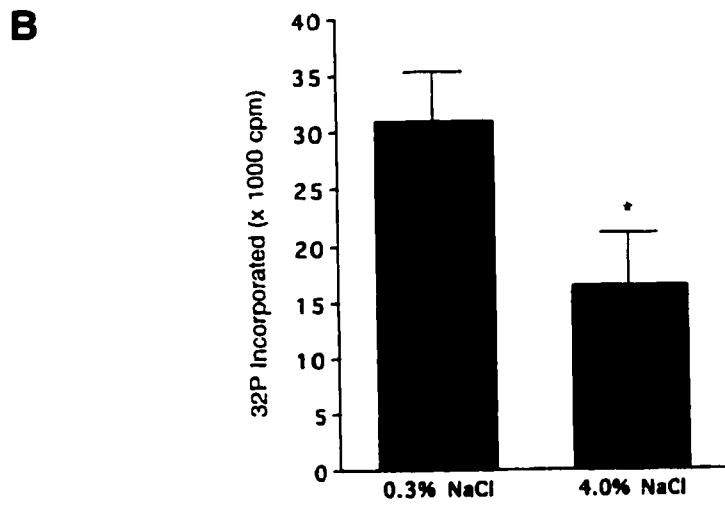
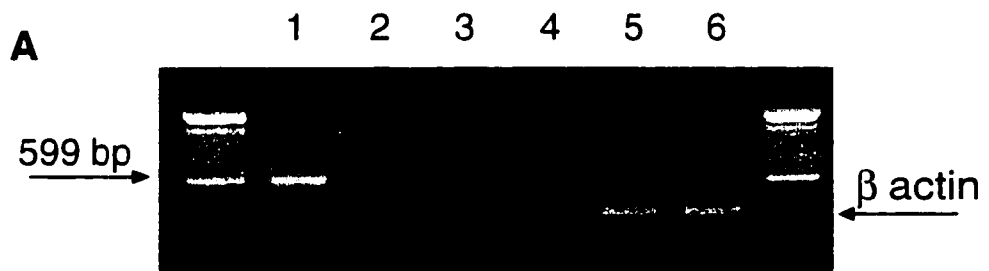
**A**



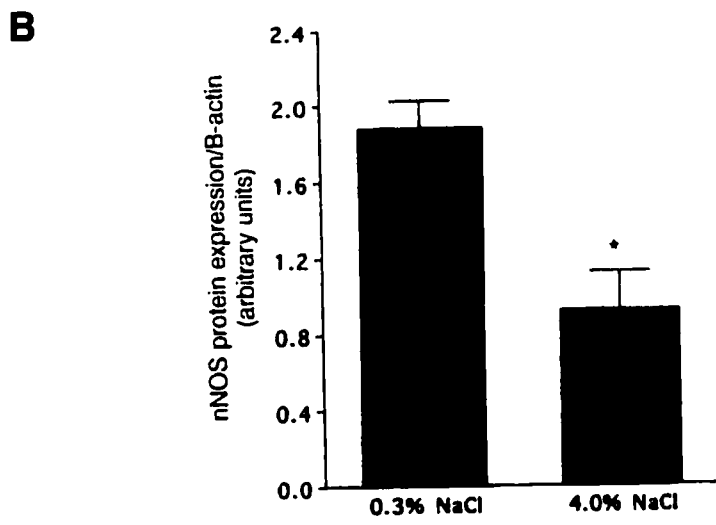
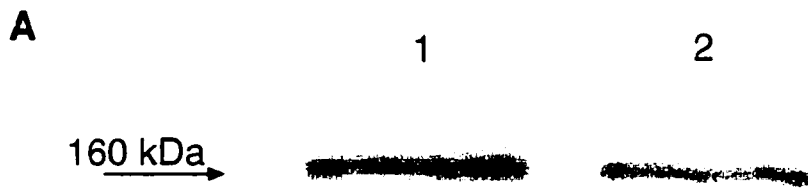
**B**



**Figure 3.7. High NaCl diet decreases nNOS mRNA expression in kidney cortex at 3 days.** (A) Representative ethidium bromide-stained agarose gel of kidney cortex nNOS RT-PCR products (599 bp) from 0.3% (lanes 1,2) and 4.0% (lanes 3,4) NaCl-fed rats. Dilutions of total RNA were (from left to right): 150 and 30 ng.  $\beta$ -actin PCR products (350) from 0.3% and 4.0% NaCl-fed rats are shown in lanes 5 and 6, respectively. (B) Bar graph depicting radioactivity incorporated into nNOS mRNA amplification products. Results are means  $\pm$  SEM, for 150 ng initial total RNA (\*  $p < 0.05$ ,  $n = 5$ ).



**Figure 3.8. High NaCl diet decreases nNOS protein expression in kidney cortex at 3 days.** (A) Representative Western blot of nNOS protein expression in kidney cortex of rats fed 4.0% NaCl diet for 3 days. Lane 1: 0.3% NaCl-fed rats (40  $\mu$ g total protein) and lane 2: 4.0% NaCl-fed rats (40  $\mu$ g total protein). (B) Bar graph depicting densitometric quantification of Western blot signals for nNOS protein from kidney cortex. Results are means  $\pm$  SEM (\*  $p < 0.05$ ,  $n = 5$ ).

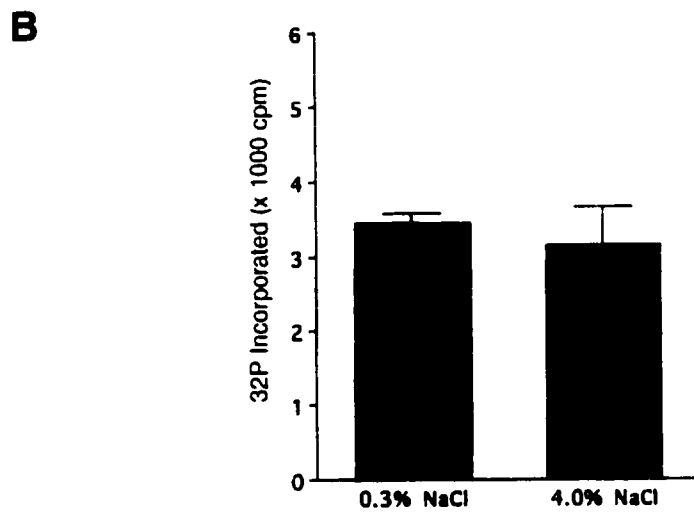
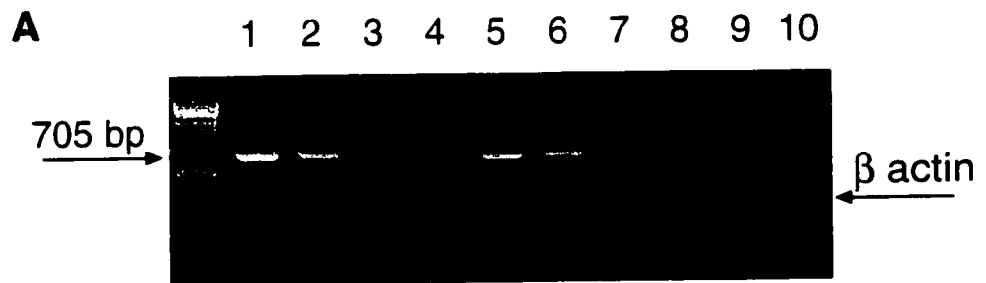


**Effect of high dietary NaCl on iNOS mRNA expression in the IMCD.**

Basal expression of iNOS mRNA has been reported in the IMCD (Mohaupt et al., 1994). High dietary NaCl intake had no effect on IMCD iNOS mRNA expression after either 3 days (Figure 3.9: 0.3% NaCl:  $3.43 \pm 0.14 \times 10^3$  vs 4.0% NaCl:  $3.14 \pm 0.52 \times 10^3$  cpm,  $p = \text{ns}$ ,  $n = 5$ ) or 3 weeks (Figure 3.10: 0.3% NaCl:  $3.90 \pm 0.32 \times 10^3$  vs 4.0% NaCl:  $4.44 \pm 0.74 \times 10^3$  cpm,  $p = \text{ns}$ ,  $n = 3$ ).

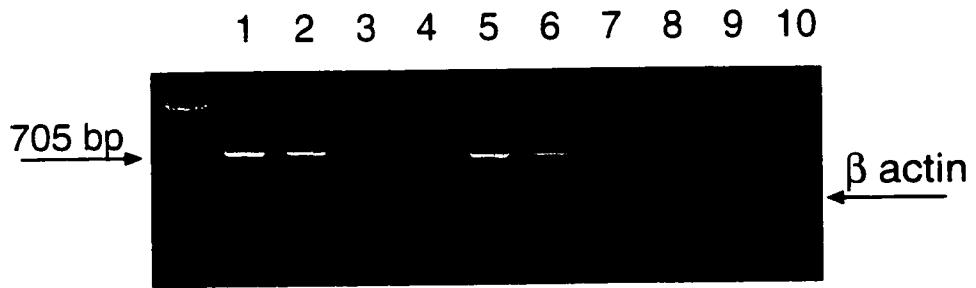
Western blots, using a mouse monoclonal antibody to iNOS (Transduction Laboratories), and with loading of up to 100  $\mu\text{g}$  protein per lane, failed to detect the expected 130 kDa iNOS protein in lysates from IMCD in either 0.3% or 4.0% NaCl-fed rats. Two other polyclonal antibodies to iNOS also did not detect the iNOS protein on Western blots.

**Figure 3.9. Effect of 3 day 4.0% NaCl diet on expression of iNOS mRNA in IMCD.** (A) Representative ethidium bromide-stained agarose gel of IMCD iNOS RT-PCR products (705 bp) from 0.3% (lanes 1-4) and 4.0% (lanes 5-8) NaCl-fed rats. Dilutions of total RNA were (from left to right): 750, 150, 30, and 6 ng.  $\beta$ -actin PCR products (350 bp) from IMCD of 0.3% and 4.0% NaCl-fed rats are shown in lanes 9 and 10, respectively. (B) Bar graph depicting radioactivity incorporated into IMCD iNOS mRNA PCR products. Results are means  $\pm$  SEM, for 6 ng initial total RNA (n= 5).

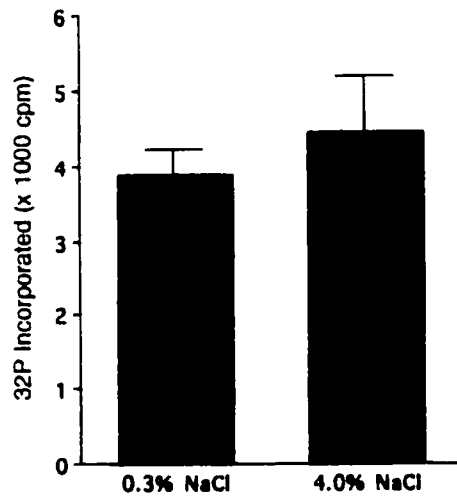


**Figure 3.10. Effect of 3 week 4.0% NaCl on expression of iNOS mRNA in IMCD.** (A) Representative ethidium bromide-stained agarose gel of IMCD iNOS RT-PCR products (705 bp) from 0.3% (lanes 1-4) and 4.0% (lanes 5-8) NaCl-fed rats. Dilutions of total RNA were (from left to right): 750, 150, 30, and 6 ng.  $\beta$ -actin PCR products (350) from IMCD of 0.3% and 4.0% NaCl-fed rats are shown in lanes 9 and 10, respectively. (B) Bar graph depicting radioactivity incorporated into IMCD iNOS PCR products. Results are means  $\pm$  SEM, for 6 ng of initial total RNA (n= 3).

**A**



**B**



## **DISCUSSION**

The present studies determined the effect of high dietary salt on nNOS expression in the IMCD. Our data clearly demonstrate that nNOS mRNA and protein are expressed in the IMCD. In addition, we show that high dietary salt intake differentially regulates nNOS mRNA and protein expression in the IMCD and renal cortex, but has no effect on expression of iNOS mRNA in the IMCD.

### **Localization of nNOS**

Utilizing RT-PCR, Terada et al. detected the highest levels of intrarenal nNOS mRNA in the IMCD (Terada et al., 1992). The results of the present study, in which we readily detected nNOS mRNA in normal rat IMCD by RT-PCR, are consistent with this study, and with the data of Singh et al. (Singh et al., 1996). Previous immunolocalization studies, however, have not detected nNOS protein expression in the IMCD, although an abundance of nNOS is present in the macula densa (Bachmann et al., 1995). In contrast, in the present studies, immunofluorescence revealed intense cytoplasmic staining for nNOS in cells of freshly microdissected IMCD, and this staining was specific for nNOS, since preincubation of the polyclonal nNOS antibody with immunizing antigen resulted in complete elimination of immunofluorescence. Furthermore, Western blot analysis of proteins extracted from IMCD revealed a single band of approximately 160 kDa that co-migrated with nNOS from a rat pituitary lysate, used as a positive control. Taken together, therefore, our data clearly demonstrate presence of nNOS mRNA and protein in IMCD. The explanation for the lack of detection of nNOS protein in IMCD by Bachmann et al. (Bachmann et al., 1995) is unclear, although we speculate that differences in either tissue processing methods, antibodies, or immunodetection methods may account for the varying results.

### **Regulation of nNOS by dietary salt in IMCD and cortex**

Inhibition of renal medullary nNOS activity increases mean arterial pressure in rats maintained on high salt diets, suggesting that this NOS isoform may be involved in the long-term regulation of blood pressure (Mattson and Bellehumeur, 1996). In the present study, nNOS protein expression in the IMCD significantly increased after three days of high dietary NaCl, but nNOS mRNA levels remained unchanged.

This suggests either increased translation of nNOS mRNA into protein, or enhanced protein stability due to post-translational modification. The regulation of nNOS gene transcription and translation, however, is incompletely understood. In this regard, nNOS mRNAs with distinct 5' untranslated regions that are encoded by separate promoters have been described in human cerebellum (Xie et al., 1995). These mRNAs likely encode identical nNOS proteins, but could differ in stability, processing, or translation efficiency. Accordingly, it is possible that high dietary salt could induce a conversion to formation of nNOS mRNA transcripts with increased efficiency of translation, via as yet undefined signaling mechanisms. Alternatively, the possibility exists that the semi-quantitative RT-PCR method utilized in the present study was not sensitive enough to detect small increases in mRNA expression that could have accounted for the increased expression of nNOS at 3 days. Our results, however, are in agreement with those of Singh et al. (Singh et al., 1996), who did not detect an effect of high salt diet for 7 days on nNOS mRNA expression in the rat IMCD, by competitive RT-PCR.

In rats on high NaCl diet for 3 weeks, IMCD nNOS mRNA and protein expression did not significantly change from control values, suggesting that the increase in nNOS protein observed at 3 days is transient. Although information regarding factors regulating nNOS expression is limited (reviewed in (Kone and Baylis, 1997)), our data suggest that the molecular signals for stimulation of nNOS protein expression are either no longer present or are counteracted by other factors after 3 weeks of high salt diet. In rat total inner medullary tissue, Mattson and Higgins reported that protein expression of eNOS, nNOS and iNOS increased significantly after 3 weeks of high dietary salt intake (Mattson and Higgins, 1996). We cannot readily provide an explanation for these differences from our present data, although it is conceivable that high dietary salt might increase nNOS expression in other inner medullary structures, such as within renal nerves, vasa recta, or inner medullary thin limbs (Bachmann et al., 1995; Terada et al., 1992). It must be noted, furthermore, that their study utilized a single monoclonal antibody to detect both nNOS and iNOS by Western blot analysis, and, in contrast to our results, they did not detect nNOS in renal cortical homogenates (Mattson and Higgins, 1996).

Within the renal cortex, the macula densa contains the highest levels of nNOS mRNA and protein (Bachmann et al., 1995; Singh et al., 1996). In rats, salt deprivation stimulates nNOS mRNA expression in the macula densa, whereas salt-loading significantly decreases mRNA expression (Bredt et al., 1991; Singh

et al., 1996). Our data on nNOS mRNA expression in renal cortex, performed as a positive control for our assay system, are in agreement with these observations. In addition, we have extended these findings by demonstrating that renal cortical nNOS protein expression is significantly down-regulated by high dietary salt intake. The cellular signaling mechanisms mediating the inverse relationship between salt intake and macula densa nNOS expression are unknown, but a recent study revealed that blockade of systemic AT<sub>2</sub> Ang II receptors significantly inhibited sodium depletion-induced increases in renal interstitial cyclic GMP levels, suggesting that AT<sub>2</sub> receptors may be linked to stimulation of renal NO production (Siragy and Carey, 1997). In addition to the possibility that differential regulation of nNOS expression between IMCD and renal cortex is due to separate cell signaling pathways, variable expression patterns may also be secondary to transcription of mRNAs from separate promoters, as discussed above (Xie et al., 1995). Furthermore, alternative splicing of nNOS mRNA yielding a novel nNOS $\mu$  protein isoform has been reported in skeletal muscle, suggesting that tissue-specific regulation of nNOS mRNA may occur (Silvagno et al., 1996).

Our data demonstrate an increase in urinary excretion of NO decomposition products after 3 days of high dietary salt intake, a time at which IMCD nNOS protein expression is increased, but renal cortical nNOS is inhibited. However, urinary excretion of NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> remained significantly increased after 3 weeks of high salt intake, despite normalization of IMCD nNOS protein expression. In rats fed a high salt diet for 2 weeks, an increase in both serum concentration and urinary excretion of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> has also been reported (Shultz and Tolins, 1993). Thus, our data suggest a dissociation between IMCD nNOS protein expression and excretion of urinary NO products. Furthermore, although absolute levels of urinary NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> were comparable in rats at 3 days and 3 weeks, we observed decreased excretion at 3 weeks, when corrected for body weight. This suggests there may be age-dependent effects on urinary excretion of NO products.

The urinary excretion of NO products is complex, representing the contributions from glomerular filtration of serum NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>, tubular handling, and *de novo* intrarenal NO synthesis. Thus, the effects of enhanced nNOS protein in IMCD on intrarenal NO production cannot be estimated from our data. The gene expression and activity of eNOS have been shown to be upregulated by shear stress (Topper et al., 1996), suggesting that increased urinary levels of NO decomposition products with high salt intake could also reflect increased intrarenal activity of this isoform. In addition, the activity of nNOS protein may be affected

by levels of intracellular calcium or endogenous inhibitors (reviewed in (Kone and Baylis, 1997)), independent of protein expression. Whether these mechanisms are involved in the regulation of nNOS activity in the IMCD with high salt is unknown.

### **Dietary NaCl and iNOS in IMCD**

Our data demonstrate tonic expression of iNOS mRNA in the IMCD of normal rats, with levels not affected by high dietary salt. Abundant iNOS mRNA has previously been detected in rat IMCD by *in situ* hybridization (Ahn et al., 1994). Restriction mapping of iNOS RT-PCR products in rat kidney revealed that the murine macrophage homologue of iNOS was the principal isoform expressed in tubular cells, including the IMCD, with lesser amounts of the rat vascular isoform of iNOS (Mohaupt et al., 1994). The present studies were not designed to determine the relative amounts of these two mRNAs in IMCD cells. Rather, we chose PCR primers to examine total iNOS transcript levels in this segment. In this regard, it is of interest that a recent study identified four alternatively spliced iNOS mRNAs in human tissues, including kidney, with upregulation of all splice variants by cytokines (Eissa et al., 1996). Accordingly, it is possible that multiple splice variants of iNOS exist in IMCD, although our data suggest that dietary salt intake does not affect total mRNA transcript levels.

In the present studies, Western blotting with 3 different antibodies failed to detect iNOS protein in IMCD. Recently, immunohistochemistry using antibodies to the macrophage-type iNOS in rat kidney demonstrated strong staining in outer medulla, cortical collecting ducts and some proximal tubules, with no detection in IMCD (Aiello et al., 1997). Tojo et al., using a vascular smooth muscle iNOS antibody in rat kidney, did not report iNOS staining in IMCD, although there was immunolabelling in terminal afferent arteriole, distal tubule and thick ascending limb (Tojo et al., 1994). Our results are in agreement with these studies, and suggest that iNOS mRNA is not efficiently translated into protein in IMCD under basal conditions or with high dietary salt.

In summary, the present studies demonstrate that cells of the normal rat IMCD express nNOS mRNA and protein. Protein expression of nNOS, but not mRNA, is upregulated by high dietary salt after 3 days. In contrast, high salt diet decreases renal cortical mRNA and protein expression of nNOS. These data

suggest that selective activation of inner medullary nNOS could be involved in the early adaptive response to high dietary salt intake.

**CHAPTER 4****Downregulation of Neuronal Nitric Oxide Synthase in the Rat Remnant Kidney**

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A. Rocznik and K. D. Burns wrote the manuscript. A. Rocznik performed all the experiments except the immunogold labelling and electron microscopy which were performed by J. N. Fryer. 5/6 nephrectomy in rats was performed according to the protocol previously established by D. Z. Levine.

**ABSTRACT**

Chronic renal failure (CRF) is associated with disturbances in nitric oxide (NO) production. We determined the effect of 5/6 nephrectomy (5/6 Nx) on expression of intrarenal neuronal nitric oxide synthase (nNOS) in the rat. In normal rat kidney, nNOS protein was detected in the macula densa and in the cytoplasm and nuclei of cells of the inner medullary collecting duct by both immunofluorescence and electron microscopy. Western blot analysis revealed that two weeks after 5/6 Nx, there were significant decreases in nNOS protein expression in renal cortex (sham:  $95.42 \pm 15.60$  vs 5/6 Nx:  $47.55 \pm 12.78$  arbitrary units,  $p < 0.05$ ,  $n = 4$ ) and inner medulla (sham:  $147.70 \pm 26.96$  vs 5/6 Nx:  $36.95 \pm 17.24$  arbitrary units,  $p < 0.005$ ,  $n = 8$ ). Losartan treatment was used to determine the role of angiotensin II (Ang II) AT<sub>1</sub> receptors in the inhibition of nNOS expression in 5/6 Nx. Losartan had no effect on the decreased expression of nNOS in the inner medulla, but partially increased nNOS protein expression in the cortex of 5/6 Nx rats. In contrast, in sham rats, losartan significantly inhibited nNOS protein expression in the cortex ( $0.66 \pm 0.04$ -fold of sham values,  $p < 0.05$ ,  $n = 6$ ) and inner medulla ( $0.74 \pm 0.12$ -fold of sham values,  $p < 0.05$ ,  $n = 6$ ). Neuronal NOS mRNA was significantly decreased in cortex and inner medulla from 5/6 Nx rats, and the effects of losartan on nNOS mRNA paralleled those observed on nNOS protein expression. These data indicate that 5/6 Nx downregulates intrarenal nNOS mRNA and protein expression. In normal rats, Ang II AT<sub>1</sub> receptors exert a tonic stimulatory effect on expression of intrarenal nNOS. We suggest that the reduction in intrarenal nNOS expression in 5/6 Nx may play a role in contributing to hypertension and altered tubular transport responses in CRF.

## INTRODUCTION

Nitric oxide (NO) regulates glomerular filtration, renal blood flow, and induces natriuresis (Kone and Baylis, 1997). NO is synthesized by a family of nitric oxide synthases (NOS), and mRNA for the neuronal NOS (nNOS) isoform is abundantly expressed in the kidney inner medulla and macula densa cells (Terada et al., 1992). Recent evidence suggests that inner medullary nNOS is involved in the regulation of sodium excretion, since inhibition of inner medullary nNOS caused development of hypertension in rats maintained on a high salt diet (Mattson and Bellehumeur, 1996). NO generated by nNOS localized in the macula densa has been postulated to regulate tubuloglomerular feedback, and intrarenal renin synthesis (Kone and Baylis, 1997).

Alterations in intrarenal NO production may be involved in the pathogenesis of chronic renal failure (CRF). In the 5/6 nephrectomized (5/6 Nx) rat, stimulation of NO production resulted in normalization of creatinine clearance, associated with increased fractional sodium excretion and decreased proteinuria, suggesting that a deficiency in NO production may be partly responsible for the impaired renal function (Ashab et al., 1995). A decrease in NOS activity in renal tissue, accompanied by a reduction in urinary excretion of NO metabolites, has been demonstrated in rats with renal mass reduction (Aiello et al., 1997). In addition, intrarenal expression of inducible NOS (iNOS) and endothelial-derived NOS (ecNOS) is decreased in 5/6 Nx rats (Aiello et al., 1997; Vaziri et al., 1998), suggesting that intrarenal NO production is impaired in CRF.

The mechanism for impaired NO production in CRF may involve the renin-angiotensin II system. Ashab et al. have shown that administration of an angiotensin converting enzyme inhibitor, captopril, to 5/6 Nx rats normalized creatinine clearance, fractional excretion of sodium, and abrogated proteinuria and hypertension. These effects were dependent on NO production, since concomitant administration of a NOS inhibitor reduced the beneficial effects of captopril (Ashab et al., 1995). Captopril also caused an increase in urinary excretion of NO products in the 5/6 Nx rat (Ashab et al., 1995). These studies support a direct relationship between angiotensin II (Ang II) and renal NO synthesis in CRF. It is of interest that in the glomeruli of 5/6 Nx rats, renin mRNA and protein content are increased, suggesting that the intrarenal

production of Ang II might be elevated (Rosenberg et al., 1991). A possible link between Ang II levels and NO production is also suggested by the work of Tojo et al who showed increased immunostaining for nNOS in the macula densa of rats fed a low salt diet (Tojo et al., 1995). In that study, however, blockade of Ang II AT<sub>1</sub> receptors with losartan was also associated with increased nNOS immunostaining. In contrast, other studies have shown that maneuvers which alter circulating levels of Ang II cause parallel changes in renal nNOS expression (Bosse et al., 1995; Schricker et al., 1996), suggesting that Ang II might directly stimulate nNOS expression.

Accordingly, we determined the effect of 5/6 Nx on expression of nNOS in the inner medulla and cortex in rats, with and without AT<sub>1</sub> receptor blockade. Our data show for the first time that 5/6 Nx suppresses expression of nNOS protein and mRNA in a fashion that is modulated by AT<sub>1</sub> receptors in the cortex but not inner medulla. Interestingly, losartan induced significant reductions in the expression of nNOS mRNA and protein in the cortex and inner medulla of sham rats, suggesting a tonic stimulatory effect of Ang II on nNOS expression in the normal kidney.

## **MATERIALS AND METHODS**

### **Animal Surgery**

Male Sprague-Dawley rats were fed regular rat chow and had unlimited access to water. Rats were anaesthetized with a mixture of oxygen (1 l/min) and halothane (gradually increased from 0.5% to 4.0%), and 5/6 Nx was performed as previously described (Levine et al., 1997). Briefly, the right kidney was exposed by midline laparotomy, and the right renal artery, vein and ureter were ligated with silk and the entire kidney removed. The poles of the left kidney were removed using a Bovie. Sham rats underwent a similar procedure, except the kidneys were only touched with the instruments. In the studies involving losartan, sham and 5/6 Nx rats received daily subcutaneous injections of losartan (25 mg/kg of body weight) (Kontogiannis and Burns, 1998) for 14 days, starting on the day of surgery. Twenty-four hours prior to sacrifice, the rats were placed in metabolic cages for urine collection. At the time of death, blood was collected from all animals for assay of blood urea nitrogen (BUN) and serum creatinine (measurements were performed by the Ottawa General Hospital Biochemistry Laboratory). After sacrifice, the left kidney was immediately removed for dissection of cortex and inner medulla. All experiments were approved by the Animal Care Committee at the University of Ottawa.

### **Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from kidney cortex and inner medulla using a commercially available kit (RNeasy, Qiagen, Chatsworth, CA). Prior to RT-PCR, residual genomic DNA was digested by incubating RNA with amplification grade DNase I (Gibco, Burlington, Ont., Canada). RNA quality was determined by running samples on 1% agarose-formaldehyde gels stained with ethidium bromide, and RNA concentration and purity were determined by optical density measurement at 260 and 280 nm. RNA samples were uniformly of high quality by these standards.

RT-PCR was performed, essentially as previously described (Roczniak et al., 1998). In preliminary experiments, serial dilutions of total RNA (6, 30, 150 and 750 ng) were prepared in RNase-free water and reverse-transcribed in a buffer containing 5 mM MgCl<sub>2</sub>, 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 1 mM each of deoxynucleoside triphosphates, 20 U RNase inhibitor, 50 U Moloney murine leukemia

virus reverse transcriptase and 2.5  $\mu$ M random hexamers (GeneAmp RNA PCR kit, Perkin-Elmer, Norwalk, CT) in a total volume of 20  $\mu$ l. After reverse transcription, the reactions were brought up to 100  $\mu$ l with 1X PCR buffer supplemented with (final concentrations) 2 mM  $MgCl_2$ , 2.5 U Ampli Taq DNA polymerase (Perkin-Elmer), and 1  $\mu$ M of nNOS-specific oligonucleotide primers. After initial denaturation at 94°C for 3 min, 33 cycles of amplification (94°C x 30 sec, 68°C x 30 sec, 72°C x 30 sec) were performed, followed by final extension at 72°C for 10 min, in a Perkin-Elmer GeneAmp PCR system 2400 apparatus. The PCR products were separated on ethidium bromide-stained 2% agarose gels for visualization. For quantification of PCR products, cDNA was resolved on gels stained with Vistra Green (Amersham, Oakville, Ont., Canada), and then subjected to phosphorimager analysis (Storm 860, Molecular Dynamics, Sunnyvale, CA).

To determine relative differences in nNOS mRNA abundance in sham and 5/6 Nx rats, we used a noncompetitive, semiquantitative PCR method as previously described (Roczniak et al., 1998). To ensure that all data were collected before PCR reactions reached a plateau phase, we measured PCR product density with a phosphorimager as a function of cycle number. Since PCR product amplification was exponential up to 40 cycles, we used 33 cycles of amplification in all experiments. Serial dilutions of total RNA from sham and 5/6 Nx rats were reverse transcribed and amplified in order to verify that all data were within the range where the logarithm of the initial amount of mRNA varies linearly with the logarithm of the amount of amplification product generated. The amplification of nNOS mRNA was linear between 6 ng and 150 ng of initial total RNA. Accordingly, in all experiments, 6 ng of total RNA from inner medulla or 30 ng from renal cortex were reverse-transcribed and PCR-amplified. To control for variations in RNA isolation and for the efficiency of the reverse transcription reaction,  $\beta$ -actin mRNA was amplified at the time of nNOS mRNA amplification. Total RNA (6 ng) was reverse-transcribed and amplified as described above, in the presence of specific primers for  $\beta$ -actin (20 nM). As a control for any possible genomic DNA contamination, a reaction lacking the reverse transcriptase enzyme was included in all experiments.

The primer sequences for nNOS and  $\beta$ -actin products were identical to those utilized in our previous studies (Roczniak et al., 1998). The primers for nNOS were chosen from the rat brain cDNA sequence

(Bredt et al., 1991). The specificity of the primers was verified through Gene-Bank.  $\beta$ -actin primers were selected according to the human cDNA sequence (Gunning et al., 1983).

The identity of the nNOS PCR product was confirmed by restriction enzyme digestion with either *Bgl* II or *Sca* I, which generated the expected products (210 and 389 bp, and 101 and 498 bp, respectively).

### **Western blotting**

Cortices and inner medullae from sham and 5/6 Nx rats were homogenized with a cell disrupter in boiling lysis buffer (1% sodium dodecyl sulphate (SDS), 10 mM Tris-HCl, pH 7.4). Western blot analysis was performed as previously described (Roczniak et al., 1998). Briefly, the lysate was boiled for 10 min, followed by centrifugation at 12,000 x g for 2 min to remove insoluble debris. Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad, Montreal, Que., Canada) using bovine serum albumin (BSA, Sigma, St. Louis, MO) as standard. Tissue lysates (20  $\mu$ g for inner medulla, and 40  $\mu$ g for cortex) were separated on 7.5% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 10% skimmed milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBS-T) and 0.01% sodium azide, for 3 hrs at room temperature. The membranes were then incubated for 18 hrs at 4°C with 1:1000 dilution of mouse monoclonal antibody to nNOS (Transduction Laboratories, Lexington, KY), followed by incubation with 1:2000 dilution of anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham). Primary antibodies were diluted in TBS-T supplemented with 5% skimmed milk and 0.01% sodium azide, whereas the secondary antibodies were diluted in TBS-T supplemented with 2% milk. Proteins were detected by enhanced chemiluminescence (ECL, Amersham) on Hyperfilm (Amersham), according to the manufacturer's instructions. Prestained standards were used as molecular weight markers (Bio-Rad), and rat pituitary protein (4  $\mu$ g, Transduction Laboratories) was used as a positive control for nNOS. To control for protein loading, all membranes were stripped and probed with a monoclonal anti- $\beta$ -actin antibody (mouse ascites fluid, Sigma), which recognized the  $\beta$ -actin protein at approximately 45 kDa. Signals on Western blots were quantified by densitometry and corrected for the  $\beta$ -actin signal, using an image analysis software program (NIH Image 1.47).

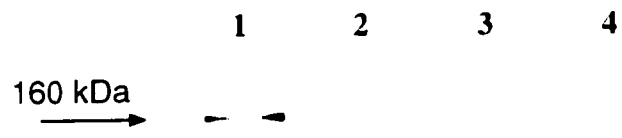
### **Immunofluorescence studies**

Animals were anaesthetized with an intraperitoneal injection of 100 mg/kg Somnotol and perfused through the abdominal aorta with 100 ml of Ringer's solution, containing 50 mM MgCl<sub>2</sub> and 1 unit/ml heparin at room temperature for 8-10 min. The animals were then perfused with Zamboni's fixative (2% paraformaldehyde, 15% picric acid in 0.1 M phosphate buffer, pH 7.3) at 4°C for 8-10 min. The left kidney was removed, rinsed in Zamboni's fixative, and kept in Zamboni's fixative for 2-3 hrs. The fixative was replaced with fresh fixative for 24 hrs at 4° C. Tissue was then rinsed in 0.1 M phosphate buffer containing 10% sucrose at 4° C. The kidneys were frozen with CO<sub>2</sub> snow to avoid freezing artifacts and tissue cracks. The tissue was then cut in a microm HM 5000 M Cryostat at 16 µm thickness, thaw-mounted onto superfrost microscope slides and stored at -80° C. Sections were allowed to thaw for 1 hr at room temperature, rinsed with phosphate-buffered saline (PBS, pH 7.4) and permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature. Slides were then incubated in blocking buffer consisting of PBS supplemented with 1% BSA for 30 min. A 1:200 dilution of polyclonal nNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the slides and incubated for 18 hrs at 4° C. To ensure specificity of staining with this antibody, additional slices were incubated in the presence of primary antibody that had been previously neutralized with the immunizing antigen (Santa Cruz Biotechnology). The slides were then washed 3 times for 5 min each in PBS and incubated with sheep anti-rabbit CY3 conjugate (1:300 dilution, Sigma-Aldrich, Oakville, Ont., Canada) for 2 hrs at room temperature. All antibodies were diluted in PBS supplemented with 0.3% Triton X-100. Following 3 washes with PBS, the slides were covered with an anti-fade compound (PBS containing 0.1 mM phenylaminediamine and 10% glycerol) and viewed with a Zeiss Axioplan fluorescent microscope (magnification x 400). Photographs were taken with Kodak Elite II color film.

The specificity of the polyclonal nNOS antibody used for immunofluorescence and immunogold was also assessed by Western blotting. As shown in Figure 4.1, this antibody recognized a 160 kDa protein corresponding to nNOS in both rat pituitary lysates and inner medullary collecting duct lysates. A very faint band of approximately 40 kDa was also seen on Westerns with inner medullary collecting duct lysates. The

polyclonal nNOS antibody did not recognize proteins from lysates from either endothelial cells or cytokine-stimulated macrophages, demonstrating that it does not cross-react with ecNOS or iNOS, respectively.

**Figure 4.1. Western blot depicting specificity of the polyclonal nNOS antibody. Lane 1: rat pituitary lysate (positive control). Lane 2: Inner medullary lysate. Lane 3: endothelial cell lysate. Lane 4: cytokine-stimulated macrophage lysate. Each lane contains 10 µg of total protein.**



### **Immunogold labeling**

Kidneys from sham rats were removed following abdominal aortic perfusion of ice-cold PBS, pH 7.4, followed by ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and 1-2-mm freehand sagittal sections were cut with a razor blade. Kidney slices were immediately immersed in fixative and then 1-mm<sup>2</sup> pieces of inner medulla were excised, immersed in fixative for 3-4 hrs, and rinsed with 0.1 M sodium phosphate buffer. Residual aldehyde groups were quenched with 50 mM glycine in 0.1 M sodium phosphate buffer for 30 min. The tissue was then rinsed with sodium phosphate buffer and treated with 0.1 M NH<sub>4</sub>Cl for 1 hr. Specimens were dehydrated sequentially in 30%, 50%, 70%, and 90% ethanol and infiltrated with 90% ethanol-LR White (Marivac, Halifax, Nova Scotia, Canada), followed by 1:2 and 1:3 mixtures, and finally by pure LR White at 4° C. The tissue samples were then placed in gelatin capsules subsequently filled with LR White and made air tight with a cover. Polymerization was performed at 50° C for 6 hrs in a vacuum oven.

Silver-gold sections were cut with a diamond knife (Marivac) and collected on formvar-coated 150-mesh nickel grids (Marivac). Sections were floated on 1% casein/ 5% normal goat serum in PBS for 30 min at room temperature to block the nonspecific binding sites, then rinsed with PBS, and treated overnight at 4° C with either 1) polyclonal nNOS antiserum (Santa Cruz Biotechnology) diluted 1:250 with PBS or 2) primary nNOS antiserum neutralized with a 10-fold excess of nNOS peptide antigen. Grids were then washed with PBS and incubated for 1 hr at room temperature with diluted (1:40) goat anti-rabbit IgG coated with 18-nm gold particles (Bio/Can Scientific, Mississauga, Ont., Canada). Sections were lightly stained with 0.25% uranyl acetate and lead citrate and examined with a Philips 300 electron microscope. Images were recorded on Eastman 35-mm film at an original magnification of x 5,750.

In immunogold sections, principal and intercalated cells of the collecting duct were readily distinguishable. Principal cells had a rectangular shape, a paucity of apical micropliae and an absence of infoldings on the basolateral membrane, while intercalated cells demonstrated a cuboidal shape, and the presence of numerous apical membrane micropliae and infoldings, a characteristic of both  $\alpha$ - and  $\beta$ -intercalated cells. Furthermore, principal cells were recognized by their relatively simple cytoplasm with

few mitochondria, whereas intercalated cells contained an abundance of cytoplasmic vesicles and mitochondria.

#### **Urinary and plasma nitrate/nitrite content**

Urinary and plasma nitrate/nitrite contents were determined by the nitrate reductase/Griess reaction assay as described (Shultz and Tolins, 1993). Plasma samples were diluted with water (1:1 vol/vol) and centrifuged through microcentrifuge filters (Centrisart, Sartorius, Gottingen, Germany) in order to remove proteins. Nitrate present in plasma filtrate and urine samples was then reduced to nitrite in the presence of nicotinamide adenine dinucleotide phosphate (150 µg/ml), flavin adenine dinucleotide (3.0 µg/ml) and 0.4 U nitrate reductase (Boehringer Mannheim, Laval, Que., Canada) for 30 min at room temperature. Nitrite was then measured by combining reduced plasma and urine aliquots with a 1:1 mixture (vol/vol) of 1% sulfanilamide (in 30% acetic acid) and 0.1% *N*-(1-naphthyl)-ethylene diamine dihydrochloride (in 60% acetic acid). Absorbencies were read on an ELISA reader at 550 nm. Nitrate and nitrite content was calculated using nitrate and nitrite standard curves, as previously reported by our laboratory (Roczniak et al., 1998).

#### **Statistics**

Results are presented as means ± SEM. Data were analyzed by either the unpaired Student T- test or the one-way analysis of variance (ANOVA) followed by Newman-Keuls test for all pair-wise comparisons. A value of  $p < 0.05$  was considered as significant.

## RESULTS

### Whole animal data

Table 4.1 summarizes values for balance and blood data in all rats studied. After 14 days, renal hypertrophy was evident in the 5/6 Nx rats; the left remnant kidney weights were significantly greater than those of sham rats, even when expressed as percent of body weight (sham:  $0.38 \pm 0.01$  vs 5/6 Nx:  $0.49 \pm 0.01$  %,  $p < 0.001$ ,  $n = 6$ ). Serum creatinine concentrations and blood urea nitrogen were also significantly increased in 5/6 Nx rats, indicating development of renal insufficiency. Urinary nitrate/nitrite excretion in 5/6 Nx rats significantly decreased compared to sham rats, whereas the plasma levels did not change (Table 4.1). Fourteen days after 5/6 Nx, systolic blood pressures in anaesthetized rats were not significantly different from those in sham animals (sham:  $139 \pm 4$  mm Hg;  $n = 13$ ; vs 5/6 Nx:  $131 \pm 4$  mm Hg;  $n = 10$ ;  $p = \text{ns}$ ), consistent with previous reports at this early time point after 5/6 Nx (Ashab et al., 1995).

### Localization of nNOS

We have previously demonstrated immunostaining for nNOS in microdissected inner medullary collecting ducts (IMCD), using a polyclonal nNOS antibody (Roczniak et al., 1998). As shown in Figure 4.2, incubation of kidney sections from sham rats with nNOS polyclonal antibody resulted in intense staining of IMCD cells. In IMCD cells, nNOS staining was observed in the cytoplasm and over the nucleus. The specificity of the labeling was verified by incubating kidney sections with nNOS antibody that had been previously neutralized with immunizing antigen, which resulted in complete loss of immunofluorescence in IMCD (not shown). Staining for nNOS was also observed in macula densa and glomerular epithelial cells, as previously described (not shown) (Bachmann et al., 1995).

To examine further the distribution of nNOS protein in IMCD cells, inner medullary sections from sham rats were processed for immunogold labeling and examined by electron microscopy. As shown in Figure 4.3A, nNOS protein was distributed throughout the cytoplasm and nuclei of IMCD principal cells. Immunogold particles for nNOS in these cells did not polarize to either apical or basolateral membrane. Cytoplasmic and nuclear staining for nNOS was also detected in intercalated cells of the initial segment of

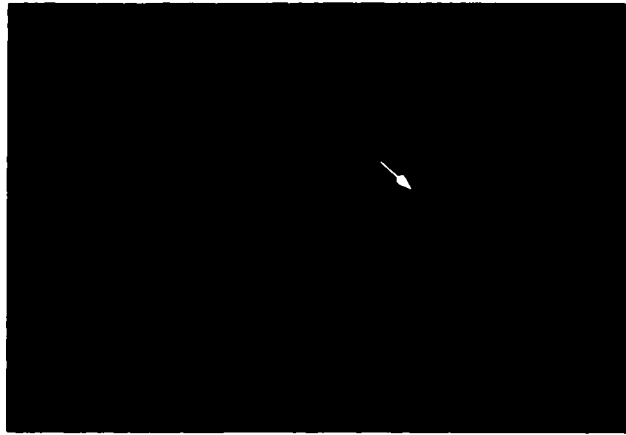
the IMCD (not shown). In contrast, inner medullary interstitial cells did not demonstrate staining in either the cytoplasm or nuclei by both immunofluorescence and immunogold. Incubation of sections with nNOS antibody previously neutralized with immunizing peptide resulted in complete loss of labeling in both principal and intercalated cells (Figure 4.3B).

**Table 4.1. Balance and blood data.**

<b>Parameter</b>	<b>Sham</b>	<b>Sham + Losartan</b>	<b>5/6 Nx</b>	<b>5/6 Nx + Losartan</b>
<b>Body wt, day of surgery (g)</b>	254.67 ± 4.54	246.50 ± 3.15	244.67 ± 3.55	235.83 ± 3.26
<b>Body wt, day 14 (g)</b>	323.50 ± 8.56	309.33 ± 6.18	310.83 ± 3.86	295.17 ± 3.74
<b>Left kidney wt (g)</b>	1.21 ± 0.03	1.18 ± 0.05	1.54 ± 0.04 <sup>b</sup>	1.45 ± 0.04 <sup>b</sup>
<b>BUN (mM)</b>	5.52 ± 0.27	6.17 ± 0.56	10.28 ± 0.46 <sup>b</sup>	11.20 ± 0.82 <sup>b</sup>
<b>Serum creatinine (μM)</b>	51.17 ± 1.28	50.00 ± 1.16	70.67 ± 1.38 <sup>b</sup>	73.00 ± 1.79 <sup>b</sup>
<b>Urinary nitrate/nitrite (μmol/100g/24 h)</b>	2.44 ± 0.11 (11)	2.33 ± 0.28 (6)	1.78 ± 0.18 <sup>c</sup> (13)	2.39 ± 0.23 (6)
<b>Plasma nitrate/nitrite (μM)</b>	44.35 ± 2.66	37.98 ± 0.71 <sup>c</sup>	40.90 ± 1.10	39.15 ± 0.84

Values are means ± SEM. <sup>b</sup>p < 0.05 vs sham or sham + losartan. <sup>c</sup>p < 0.05 vs sham. Values in parentheses represent number of experiments, n = 6 for all other measurements. See text for details.

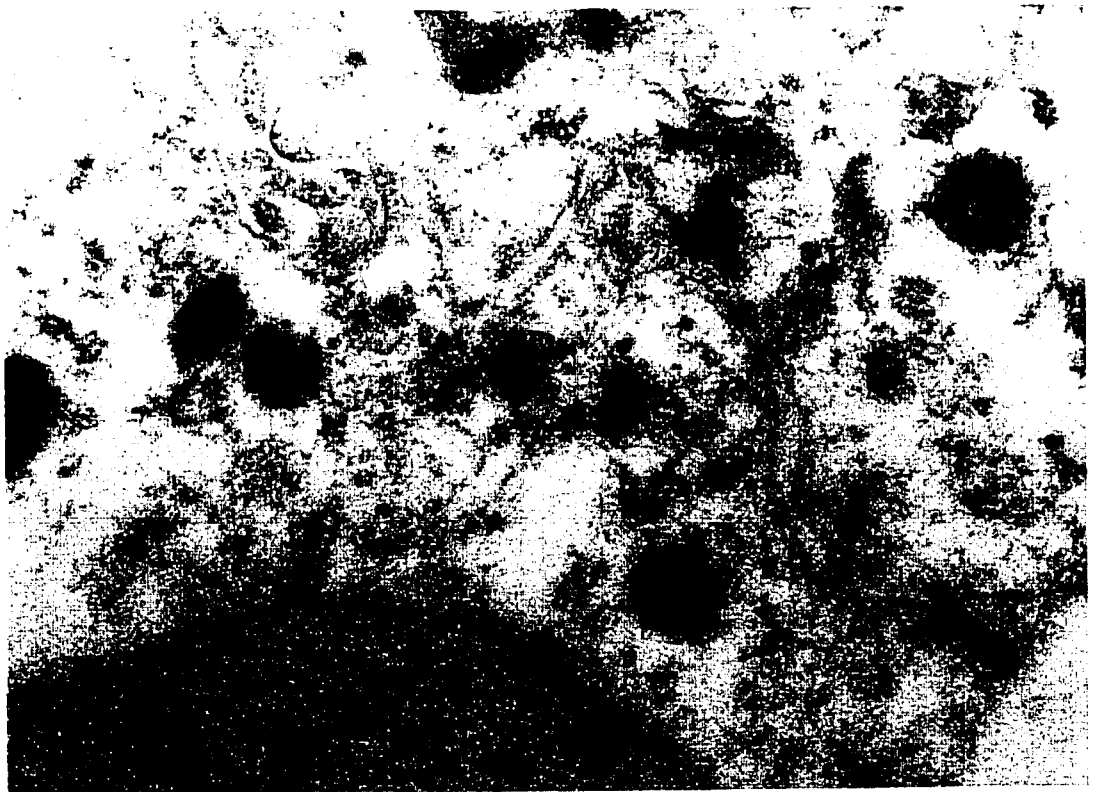
**Figure 4.2. Immunolocalization of nNOS.** Immunofluorescence micrographs of IMCD (arrow) labeled with polyclonal nNOS antibody (magnification x 400).



**Figure 4.3. Immunogold localization of nNOS.** Electron micrograph of IMCD principal cell labeled with polyclonal nNOS antibody (**A**). Grains are observed in cytoplasm (arrow) and nucleus (arrowhead). IMCD principal cell labeled with nNOS polyclonal antibody neutralized by preincubation with immunizing antigen is shown in **B** (magnification x 52,800).



A



**Effect of 5/6 Nx on expression of nNOS protein in the cortex and inner medulla.**

The effect of 5/6 Nx on the expression of nNOS protein in the cortex and inner medulla was determined by Western blotting. Fourteen days after 5/6 Nx, nNOS protein expression was significantly decreased in the cortex (Figure 4.4: sham:  $95.42 \pm 15.60$  vs 5/6 Nx:  $47.55 \pm 12.78$  arbitrary units,  $p < 0.05$ ,  $n = 4$ ) and in the inner medulla (Figure 4.5: sham:  $147.70 \pm 26.96$  vs 5/6 Nx:  $36.95 \pm 17.24$  arbitrary units,  $p < 0.005$ ,  $n = 8$ ).

**Effect of losartan on the expression of nNOS protein in the cortex and inner medulla.**

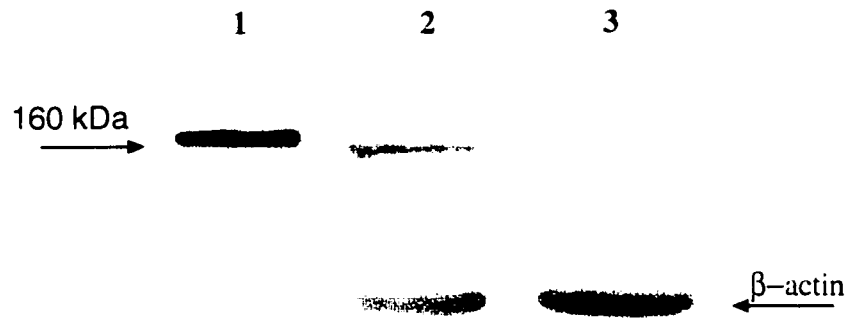
To determine the role of AT<sub>1</sub> Ang II receptors in the downregulation of nNOS protein in the cortex and inner medulla of 5/6 Nx rats, 5/6 Nx and sham rats were injected daily with either losartan (25 mg/kg, s.c.) or vehicle (saline), starting on the day of surgery. Losartan had no effect on kidney weights, BUN, or serum creatinine after 14 days (Table 4.1). In 5/6 Nx rats, losartan increased urinary nitrite/nitrate excretion to the levels observed in sham rats. In sham rats, losartan decreased plasma nitrite/nitrate content (Table 4.1).

In 5/6 Nx rats, administration of losartan caused a small but significant increase in nNOS protein expression in the renal cortex (Figure 4.6: 5/6 Nx:  $0.05 \pm 0.01$  vs 5/6 Nx + losartan:  $0.15 \pm 0.04$ -fold of sham levels,  $p < 0.05$ ,  $n = 6$ ). In contrast to renal cortex, administration of losartan did not alter the inhibitory effect of 5/6 Nx on nNOS protein expression in the inner medulla (Figure 7: 5/6 Nx:  $0.28 \pm 0.06$  vs 5/6 Nx + losartan:  $0.23 \pm 0.05$ -fold of sham levels,  $p = \text{ns}$ ,  $n = 6$ ).

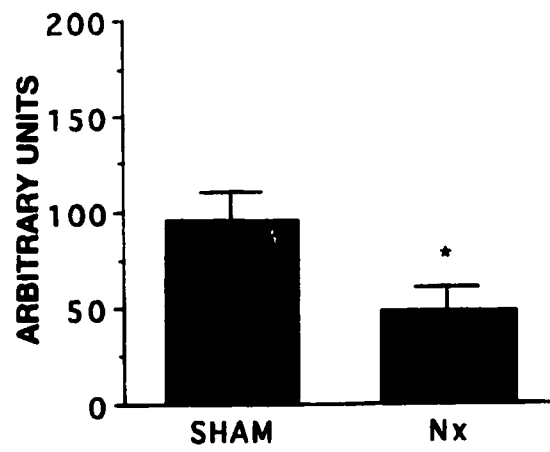
Administration of losartan to sham rats significantly reduced nNOS protein expression in the cortex (Figure 4.6: sham + losartan:  $0.66 \pm 0.04$ -fold of sham levels,  $p < 0.05$ ,  $n = 6$ ) and in the inner medulla (Figure 4.7: sham + losartan:  $0.74 \pm 0.12$ - fold of sham levels,  $p < 0.05$ ,  $n = 6$ ).

**Figure 4.4. Effect of 5/6 Nx on cortical expression of nNOS protein.** (A) Representative Western blot of nNOS protein expression in the cortex of sham and 5/6 Nx rats. Lane 1: rat pituitary lysate (positive control). Lane 2: 40  $\mu$ g total protein from sham rat. Lane 3: 40  $\mu$ g total protein from 5/6 Nx rat. Corresponding bands for  $\beta$ -actin are depicted, demonstrating equal loading of proteins. (B) Bar graph depicting densitometric quantification of Western blot signals for nNOS protein in the cortex. Results are means  $\pm$  SEM (n= 4), \* p< 0.05.

**A**

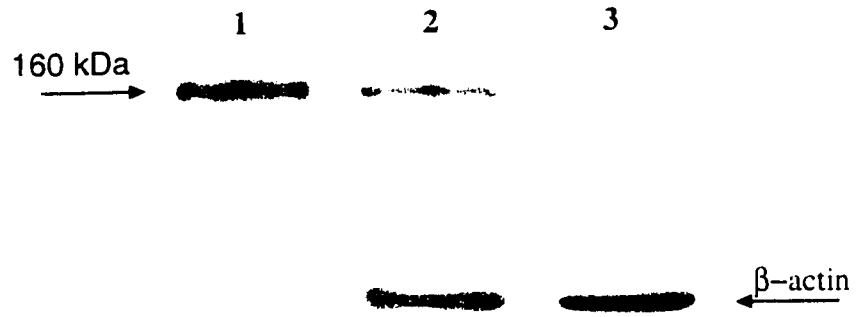


**B**

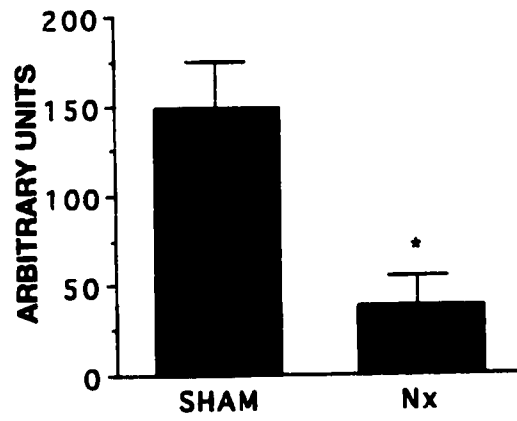


**Figure 4.5. Effect of 5/6 Nx on inner medullary expression of nNOS protein. (A)** Representative Western blot of nNOS protein expression in the inner medulla of sham and 5/6 Nx rats. Lane 1: rat pituitary lysate (positive control). Lane 2: 20  $\mu$ g total protein from sham rat. Lane 3: 20  $\mu$ g total protein from 5/6 Nx rat. Corresponding bands for  $\beta$ -actin are depicted, demonstrating equal loading of proteins. **(B)** Bar graph depicting densitometric quantification of Western blot signals for nNOS protein in the inner medulla. Results are means  $\pm$  SEM (n= 8), \* p< 0.005.

**A**

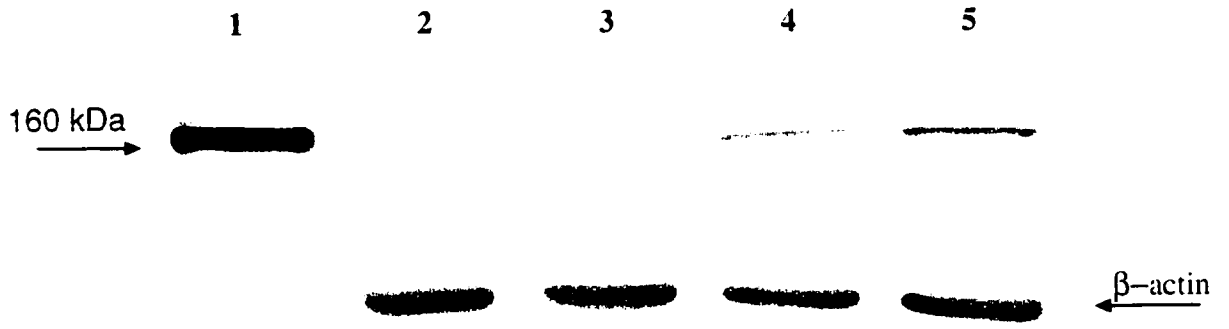


**B**

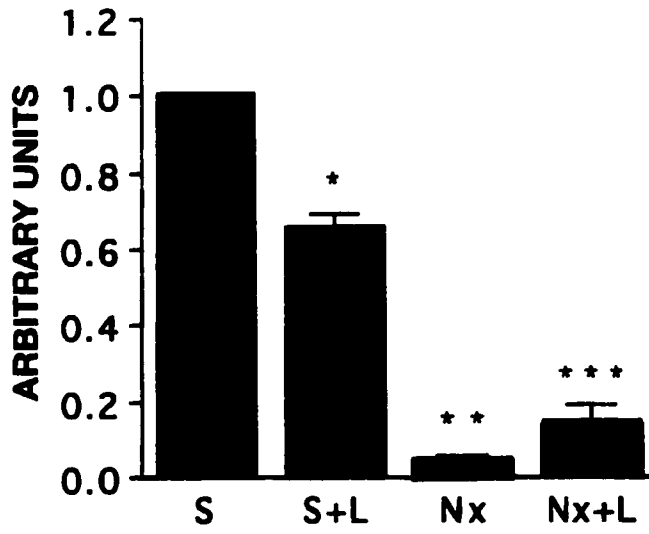


**Figure 4.6. Effect of losartan on cortical nNOS protein expression.** (A) Representative Western blot of nNOS protein expression in the cortex of sham and 5/6 Nx rats. Lane 1: rat pituitary lysate (positive control). Lane 2: 5/6 Nx + losartan. Lane 3: 5/6 Nx. Lane 4: sham + losartan. Lane 5: sham. Each lane contains 40  $\mu$ g total protein. Corresponding bands for  $\beta$ -actin are depicted, demonstrating equal loading of proteins. (B) Bar graph depicting densitometric quantification of Western blot signals for nNOS protein in the cortex. S: sham, S + L: sham + losartan, Nx: 5/6 Nx, and Nx + L: 5/6 Nx + losartan. Results are means  $\pm$  SEM (n= 6), \* p< 0.05 vs sham, \*\* p< 0.05 vs sham and \*\*\* p< 0.05 vs 5/6 Nx.

**A**

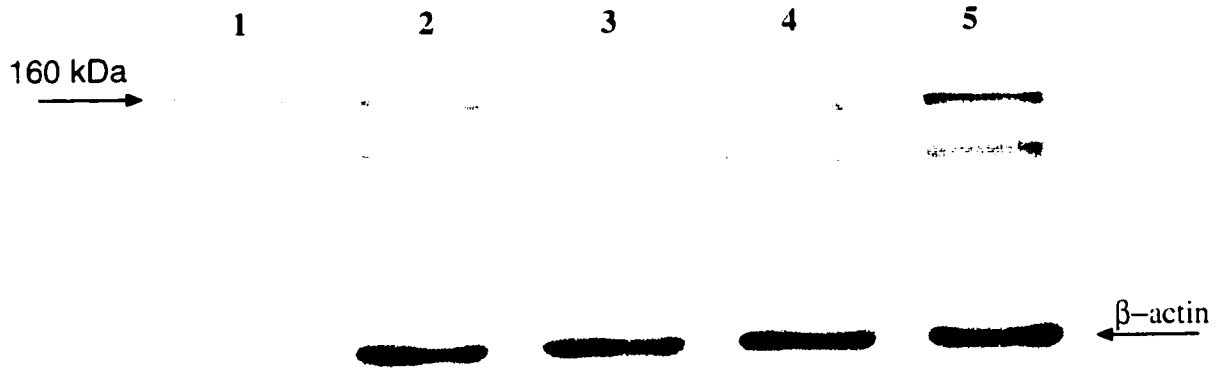


**B**

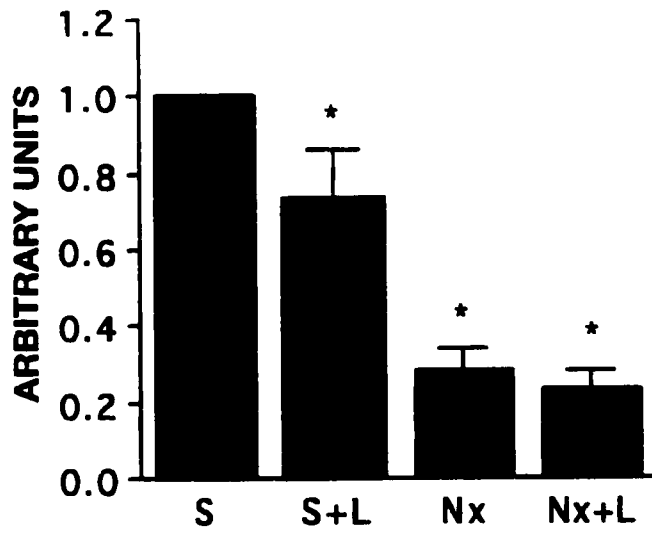


**Figure 4.7. Effect of losartan on inner medullary nNOS protein expression.** (A) Representative Western blot of nNOS protein expression in the inner medulla of sham and 5/6 Nx rats. Lane 1: rat pituitary lysate (positive control). Lane 2: 5/6 Nx + losartan. Lane 3: 5/6 Nx. Lane 4: sham + losartan. Lane 5: sham. Each lane contains 40  $\mu$ g total protein. Corresponding bands for  $\beta$ -actin are depicted, demonstrating equal loading of proteins. (B) Bar graph depicting densitometric quantification of Western blot signals for nNOS protein in the inner medulla. S: sham, S + L: sham + losartan, Nx: 5/6 Nx, and Nx + L: 5/6 Nx + losartan. Results are means  $\pm$  SEM (n= 6), \* p< 0.05 vs sham.

**A**



**B**



**Expression of nNOS mRNA in the cortex and inner medulla.**

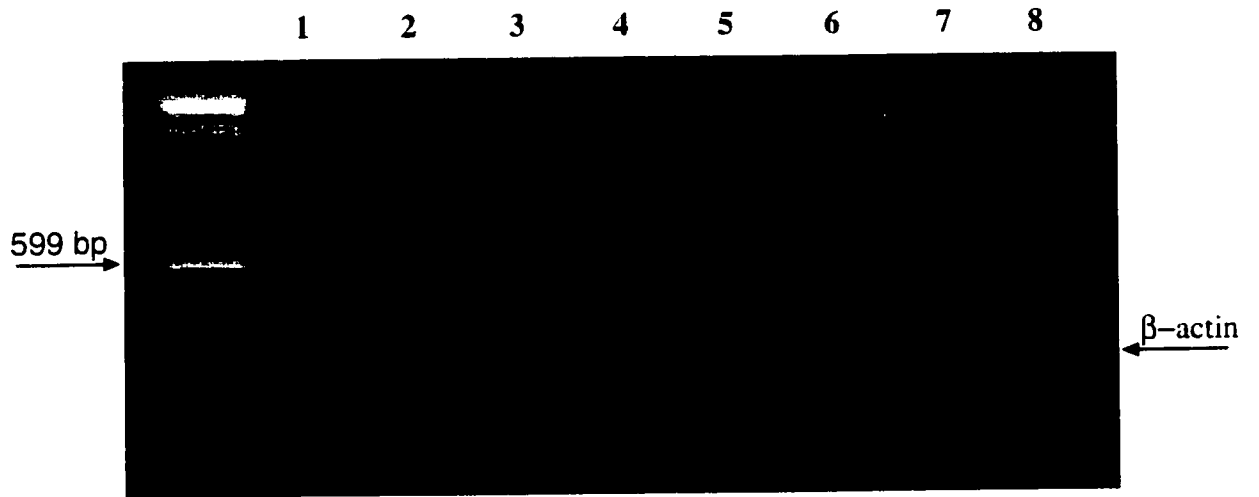
In 5/6 Nx rats, changes in mRNA expression for nNOS in cortex and inner medulla were concordant with alterations in protein expression. In the cortex, nNOS mRNA expression significantly decreased in 5/6 Nx rats (Figure 4.8: 5/6 Nx:  $0.43 \pm 0.07$ -fold of sham levels,  $p < 0.05$ ,  $n = 6$ ). As with cortical nNOS protein expression, this inhibitory effect was partially reversed by losartan (5/6 Nx + losartan:  $0.72 \pm 0.12$ -fold of sham levels,  $p < 0.05$  vs 5/6 Nx,  $n = 6$ ). In sham rats, losartan administration significantly decreased nNOS mRNA expression in the cortex (sham + losartan:  $0.69 \pm 0.08$ -fold of sham level,  $p < 0.05$ ,  $n = 6$ ).

In the inner medulla of 5/6 Nx rats, nNOS mRNA was also significantly downregulated (Figure 4.9: 5/6 Nx:  $0.42 \pm 0.19$ -fold of sham level,  $p < 0.05$ ,  $n = 6$ ). Losartan did not reverse this inhibitory effect (5/6 Nx + losartan:  $0.30 \pm 0.08$ -fold of sham level,  $p = ns$  vs 5/6 Nx,  $n = 6$ ). However, administration of losartan significantly reduced nNOS mRNA levels in the inner medulla of sham rats (sham + losartan:  $0.59 \pm 0.16$ -fold of sham level,  $p < 0.05$ ,  $n = 6$ ).

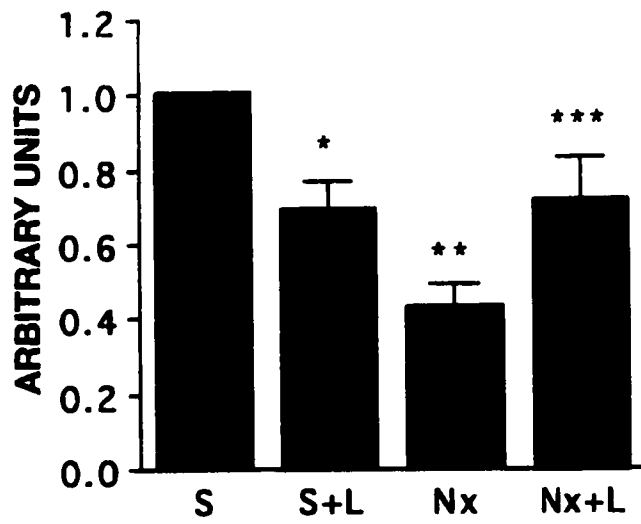
In all experiments, to control for possible variations in RNA isolation and reverse transcription reactions,  $\beta$ -actin mRNA was PCR-amplified along with the nNOS mRNA amplifications. There were no significant differences in the intensities of  $\beta$ -actin PCR products between samples derived from sham or 5/6 Nx rats (not shown). Similarly, there were no differences in  $\beta$ -actin protein abundance between samples derived from either sham or 5/6 Nx rats, by Western blot analysis (not shown).

**Figure 4.8. Effect of 5/6 Nx and losartan on cortical nNOS mRNA expression.** (A) Representative ethidium bromide-stained agarose gel of cortical nNOS cDNA product (599 bp). 30 ng of total RNA was reverse-transcribed and amplified by PCR. Lane 1: 5/6 Nx + losartan. Lane 2: 5/6 Nx. Lane 3: sham + losartan. Lane 4: sham.  $\beta$ -actin PCR products (350 bp) corresponding to lanes 1, 2, 3 and 4 are shown in lanes 5, 6, 7 and 8 respectively. (B) Bar graph depicting quantity of nNOS mRNA amplification products determined by phosphorimager analysis. S: sham, S + L: sham + losartan, Nx: 5/6 Nx, and Nx + L: 5/6 Nx + losartan. Results are means  $\pm$  SEM (n= 6), \* p< 0.05 vs sham, \*\* p< 0.05 vs sham and \*\*\* p< 0.05 vs 5/6 Nx.

A



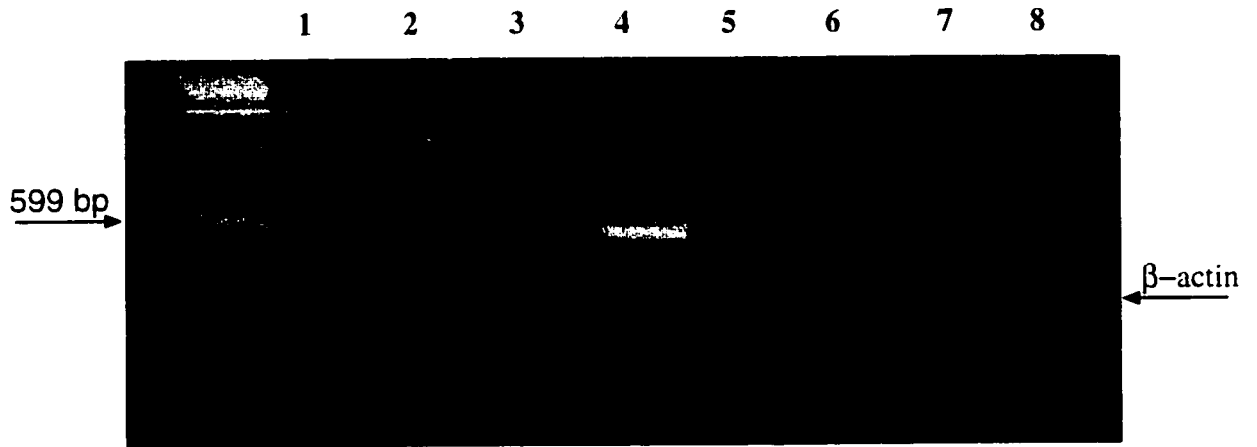
B



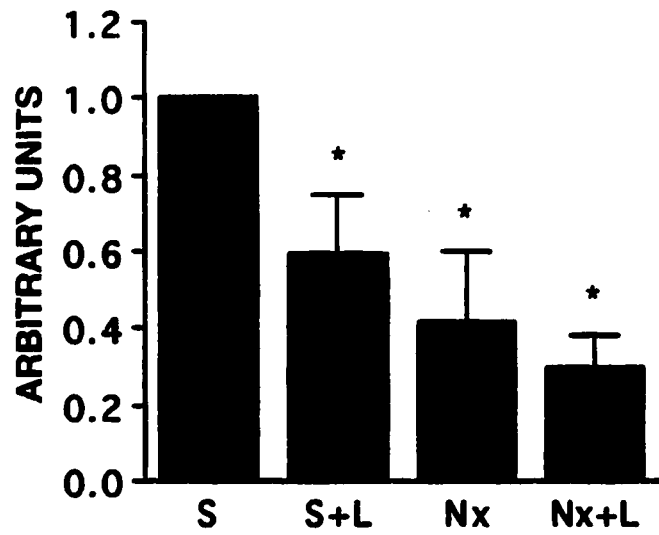
**Figure 4.9. Effect of 5/6 Nx and losartan on inner medullary nNOS mRNA expression. (A)**

Representative ethidium bromide-stained agarose gel of inner medullary nNOS cDNA product (599 bp). 6 ng of total RNA was reverse-transcribed and amplified by PCR. Lane 1: 5/6 Nx + losartan. Lane 2: 5/6 Nx. Lane 3: sham + losartan. Lane 4: sham.  $\beta$ -actin PCR products (350 bp) corresponding to lanes 1, 2, 3 and 4 are shown in lanes 5, 6, 7 and 8 respectively. **(B)** Bar graph depicting quantity of nNOS mRNA amplification products determined by phosphorimager analysis. S: sham, S + L: sham + losartan, Nx: 5/6 Nx, and Nx + L: 5/6 Nx + losartan. Results are means  $\pm$  SEM (n=6), \* p< 0.05 vs sham.

A



B



## DISCUSSION

The major finding of the present studies is that nNOS mRNA and protein expression is downregulated in the cortex and inner medulla of 5/6 Nx rats. In the normal kidney, our data demonstrate a relative abundance of nNOS protein in inner medullary collecting duct and macula densa cells. Within the inner medullary collecting duct, nNOS was detected in cytoplasm and over cell nuclei, by both immunofluorescence and immunogold electron microscopy. Administration of losartan only partially reversed the inhibitory effect of 5/6 Nx on the expression of nNOS in the cortex, but had no effect in the inner medulla. Interestingly, losartan significantly downregulated the expression of nNOS mRNA and protein in both cortex and inner medulla of sham rats, indicating that Ang II exerts a tonic stimulatory effect on intrarenal nNOS expression in the normal rat.

### **The effect of 5/6 Nx on nNOS expression**

In rats with renal mass reduction, both renal and systemic NO synthesis are reduced (Aiello et al., 1997). This may be of pathophysiologic importance, since stimulation of NO production by administration of L-arginine to 5/6 Nx rats reduced proteinuria and normalized creatinine clearance, associated with an increase in fractional excretion of sodium (Ashab et al., 1995). In the present studies, we observed that 2 weeks after 5/6 Nx, urinary excretion of NO metabolic products was significantly reduced, whereas plasma levels of these products remained constant. Since urinary excretion of nitrate/nitrite is dependent on glomerular filtration, tubular handling and *de novo* intrarenal synthesis of NO, it is difficult to speculate on the significance of urinary levels of these products in this model of CRF. Our results are, however, in agreement with the work of Vaziri et al. (Vaziri et al., 1998), and are at least suggestive that systemic NO production is reduced in CRF, since plasma levels of nitrate/nitrite did not increase with impaired GFR.

In order to determine whether impaired intrarenal NO synthesis might contribute to decreased urinary nitrate/nitrite excretion in CRF, we measured mRNA and protein expression for nNOS in cortex and inner medulla of 5/6 Nx rats, by RT-PCR and Western blotting, respectively. Our data show that nNOS mRNA and protein expression in both kidney regions is significantly downregulated in 5/6 Nx rats. The potential significance of a reduction in renal nNOS activity in CRF is suggested by the reported

intrarenal actions of NO derived from nNOS. In the cortex, for example, nNOS modulates afferent arteriolar tone in response to changes in tubular fluid flow (tubuloglomerular feedback) and regulates the responsiveness of efferent arteriolar tone to Ang II (Ichihara et al., 1998). Neuronal NOS activity may also regulate renin synthesis by juxtaglomerular cells (Kone and Baylis, 1997). Therefore, reduced cortical NO synthesis secondary to decreased nNOS expression might contribute to either increased intraglomerular pressures, or to the loss of afferent arteriolar autoregulatory function in CRF (Hostetter et al., 1981). On the other hand, loss of inner medullary nNOS in CRF might be expected to contribute to excessive sodium retention and eventual hypertension, since studies in rats fed high salt diets indicate that inhibition of nNOS expression in the inner medulla causes enhanced sodium reabsorption and elevated blood pressures (Mattson and Bellehumeur, 1996).

#### **Effect of losartan on nNOS expression**

Interactions between NO and Ang II are critical in regulating renal hemodynamics and excretory function (Deng et al., 1996; Ichihara et al., 1998; Kone and Baylis, 1997). Studies in salt depleted and salt fed rats indicate that nNOS mRNA expression in the macula densa varies in parallel with Ang II levels, suggesting that at least in the macula densa Ang II may increase nNOS expression (Singh et al., 1996). In contrast, high dietary salt increases nNOS protein expression in IMCD (Roczniak et al., 1998).

To test the hypothesis that the 5/6 Nx-induced downregulation of nNOS expression in the cortex and inner medulla is mediated by Ang AT<sub>1</sub> receptors, sham and 5/6 Nx rats were treated with losartan. Our data show that losartan only partly reversed nNOS mRNA and protein expression in the cortex, and had no effect on nNOS in the inner medulla. Although losartan reversed cortical nNOS mRNA expression to levels comparable to those in losartan-treated sham rats, cortical nNOS protein expression remained markedly decreased. Although the mechanism for this effect of losartan is unclear, one possibility may be that mRNA expression of cortical nNOS in CRF is inhibited by Ang II AT<sub>1</sub> receptor activation, whereas translation of nNOS protein remains blocked, in a manner independent of Ang II AT<sub>1</sub> receptors. Mediators of the inhibitory effect of 5/6 Nx on nNOS expression in the cortex and the inner medulla are unknown, but may include growth factors, cytokines or endothelin-1, all of which have been reported to be increased in CRF (Terzi et al., 1998). Furthermore, a recent study demonstrated that increased parathyroid hormone

(PTH) levels in 5/6 Nx rats caused a downregulation of renal expression of both iNOS and ecNOS (Vaziri et al., 1998). The effect of PTH on intrarenal nNOS expression in CRF, however, is unknown.

Although losartan had no effect on urinary nitrate/nitrite excretion in normal rats, it increased NO metabolic product excretion in 5/6 Nx rats to levels not different from those in sham rats. These data are consistent with those of Ashab et al, who reported a stimulatory effect of captopril on urinary nitrate/nitrite in 5/6 Nx rats (Ashab et al., 1995). In the present studies, losartan did not affect serum creatinine concentrations, indicating that the stimulatory effect of losartan on nitrate/nitrite excretion in 5/6 Nx rats is independent of GFR. Although our data suggest a contribution of increased cortical nNOS protein expression to urinary nitrate/nitrite excretion with losartan treatment, it is also possible that other NOS isoforms are affected by AT<sub>1</sub> receptor blockade. Ang II has been reported to inhibit renal iNOS expression (Wolf et al., 1997), suggesting that iNOS activity might be enhanced by losartan in 5/6 Nx rats. With respect to plasma levels of NO metabolic products, we observed that losartan had no effect in 5/6 Nx rats, but caused a slight but significant decrease in normal rats. The mechanism for this effect is unclear, although it is of interest that Ang II has been reported to stimulate gene expression of the ecNOS isoform (Hennington et al., 1998). Thus, it is possible that losartan downregulates ecNOS expression in the systemic vasculature, an effect limited to the normal rat.

Our data show that losartan decreased nNOS mRNA and protein expression in both cortex and inner medulla of sham rats, suggesting that in normal kidney, Ang II tonically stimulates intrarenal nNOS expression by acting on AT<sub>1</sub> receptors. Indeed, in normal rats, infusion of Ang II increased NO production in the inner medulla, suggesting an important role for Ang II-stimulated NO production in modulating medullary hemodynamics (Zou and Cowley, 1997). In contrast to our data, Kurtz et al showed that renal levels of nNOS mRNA did not change after normal rats were treated with losartan, although in that study losartan was only administered for two days (Schricker et al., 1996). In contrast to the normal kidney, our results indicate that Ang II AT<sub>1</sub> receptor blockade stimulates renal cortical nNOS expression in the 5/6 Nx rat. As noted above, the mechanism for this is unclear. It is possible that AT<sub>1</sub> receptor signalling is altered in the remnant kidney, or that 5/6 Nx might stimulate expression of intrarenal AT<sub>2</sub> receptors, postulated to be linked to renal NO production (Siragy and Carey, 1997). Thus, losartan could elevate circulating levels

of Ang II (Goldberg et al., 1993), which could then act on AT<sub>2</sub> receptors in the remnant kidney to increase NO production. Further studies are required to test this hypothesis.

In summary, 5/6 nephrectomy causes a significant downregulation of intrarenal nNOS mRNA and protein expression. Ang II AT<sub>1</sub> receptors exert a stimulatory effect on intrarenal expression of nNOS in the normal kidney, and are partly responsible for the downregulation of nNOS expression in the cortex of 5/6 nephrectomized rats. The reduction in intrarenal nNOS expression in 5/6 Nx suggests a role for nNOS in inducing hypertension and altered tubular transport responses in chronic renal failure.

**CHAPTER 5****Localization of Protein Inhibitor of Neuronal Nitric Oxide Synthase (PIN)  
in Rat Kidney**

Agnes Roczniak, David Z. Levine and Kevin D. Burns

*Am. J. Physiol. (submitted)*

A. Roczniak and K. D. Burns wrote the paper. A. Roczniak performed all the experiments. 5/6 nephrectomy was performed according to the protocol previously established by Dr D. Z. Levine.

**ABSTRACT**

We have recently demonstrated that in rats with 5/6 nephrectomy (5/6 Nx), renal cortical and inner medullary neuronal NOS (nNOS) expression is downregulated, associated with decreased urinary excretion of nitric oxide (NO) products. Recently, a novel 89 amino acid protein (PIN- protein inhibitor of nNOS) was isolated from rat brain and shown to inhibit nNOS activity. The present studies localized PIN in the rat kidney and determined the effect of 5/6 Nx on PIN expression. By reverse transcription polymerase chain reaction, PIN mRNA was detected in the kidney cortex and inner medulla. Immunohistochemistry revealed staining for PIN in glomerular and vasa rectae endothelial cells. PIN was also localized to the apical membranes of inner medullary collecting duct (IMCD) cells. Two weeks after 5/6 Nx, inner medullary PIN expression was significantly upregulated (sham:  $0.18 \pm 0.07$  vs. 5/6 Nx:  $0.58 \pm 0.13$  arbitrary units,  $n=6$ ,  $p<0.02$ ), as determined by Western blotting. In summary, our data show that PIN, a specific inhibitor of nNOS activity, is expressed in the IMCD, a site of high nNOS expression in the kidney. PIN expression is upregulated in the inner medulla of 5/6 Nx rats. Inhibition of nNOS activity by PIN may have important implications for the regulation of NO synthesis in the IMCD of normal and remnant kidneys.

## INTRODUCTION

Nitric oxide (NO) is generated in the kidney by the conversion of L-arginine to L-citrulline, in a reaction catalyzed by members of the nitric oxide synthase (NOS) family (Kone and Baylis, 1997). The neuronal nitric oxide synthase (nNOS) is highly expressed in the macula densa and inner medullary collecting duct (IMCD) of the kidney (Bachmann et al., 1995; Rocznik et al., 1998). Nitric oxide generated by nNOS in the macula densa has been implicated in the regulation of tubuloglomerular feedback and renin secretion (Kurtz et al., 1998), whereas nNOS in the inner medulla may be involved in the long-term regulation of arterial blood pressure (Mattson and Bellehumeur, 1996).

The activity of nNOS is regulated by  $\text{Ca}^{2+}$ /calmodulin binding, phosphorylation, and feedback inhibition by NO (Kone and Baylis, 1997). Another potential mechanism of nNOS regulation has recently been identified. Using the yeast two-hybrid system, Jaffrey et al. isolated a novel 89 amino acid protein termed protein inhibitor of nNOS (PIN), from rat hippocampus (Jaffrey and Snyder, 1996). PIN is a highly conserved protein across species, and shares 100% homology with the light chain component of dynein (Dick et al., 1996; Guo et al., 1999). In HEK293 cells, PIN inhibits calcium ionophore-stimulated cGMP formation and nNOS activity, in a concentration-dependent manner (Jaffrey and Snyder, 1996). The mechanism of PIN-induced inhibition of nNOS activity involves its binding to a 17-amino acid sequence of nNOS, causing destabilization of the dimeric structure of nNOS (Fan et al., 1998). Inhibition of nNOS activity by PIN appears to be highly specific, since the PIN binding domain of nNOS (amino acid residues 161-245) is absent from both the endothelial and inducible NOS (ecNOS and iNOS, respectively) (Fan et al., 1998).

Chronic renal failure (CRF) is characterized by decreased intrarenal NO production, which may contribute to elevation of blood pressure (Ashab et al., 1995). Mechanisms that may contribute to reduction of renal NO production include downregulation of renal nNOS, ecNOS and iNOS expression (Rocznik et al., 1999; Vaziri et al., 1998). Indeed, in a recent study, we demonstrated that 5/6 nephrectomy (5/6 Nx) in rats inhibits expression of nNOS mRNA and protein in the renal cortex and inner medulla (Rocznik et al., 1999).

Despite the discovery of an endogenous protein inhibitor of nNOS, little is known about its distribution and regulation in the kidney. Accordingly, the first objective of our study was to localize PIN expression in the kidney. Secondly, we determined if PIN expression is altered in 5/6 Nx rats. Our data show that PIN is highly expressed in the endothelial cells of glomeruli, afferent arterioles and vasa rectae, whereas in the inner medulla, labeling of the apical surface of IMCD cells was observed. In 5/6 Nx rats, inner medullary PIN expression is significantly upregulated, suggesting that PIN could contribute to a decrease in NO synthesis by this segment.

## **MATERIALS and METHODS**

### **Animal Surgery**

Male Sprague-Dawley rats (200-250 g) were fed regular rat chow and had unlimited access to water. Rats were anaesthetized with a mixture of oxygen (1 l/min) and halothane (gradually increased from 0.5% to 4.0%), and 5/6 Nx was performed as previously described (Roczniak et al., 1999). Briefly, the right kidney was exposed by midline laparotomy, and the right renal artery, vein and ureter were ligated with silk and the entire kidney removed. The poles of the left kidney were removed using a Bovie. Sham rats underwent a similar procedure, except the kidneys were only touched with the instruments. Two weeks after 5/6 Nx, rats were sacrificed and the left kidney immediately removed for dissection of cortex and inner medulla, or used for immunohistochemistry. All experiments were approved by the Animal Care Committee at the University of Ottawa.

### **Reverse transcription- polymerase chain reaction (RT-PCR)**

Total RNA was isolated from kidney cortex and inner medulla using a commercially available kit (RNeasy, Qiagen, Chatsworth, CA). Prior to RT-PCR, residual genomic DNA was digested by incubating RNA with amplification grade DNase I (Gibco, Burlington, Ont., Canada). RNA quality was determined by running samples on 1% agarose-formaldehyde gels stained with ethidium bromide, and RNA concentration and purity were determined by optical density measurement at 260 and 280 nm. RNA samples were uniformly of high quality by these standards.

RT-PCR was performed, essentially as described (Roczniak et al., 1999). 100 ng of total RNA was reverse-transcribed and amplified in the presence of 1  $\mu$ M of PIN-specific oligonucleotide primers. After initial denaturation at 94°C for 3 min, 40 cycles of amplification (94°C x 30 sec, 60°C x 30 sec, 72°C x 30 sec) were performed, followed by final extension at 72°C for 10 min, in a Perkin-Elmer GeneAmp PCR system 2400 apparatus. The PCR products were separated on ethidium bromide-stained 2% agarose gels, visualized and photographed under UV light.

The primer sequences for PIN were selected according to the published rat cDNA sequence (Jaffrey and Snyder, 1996). The upstream primer was: 5'- GTAACCATGTGCGACCGGAA -3' (bases: 91 - 110), and the downstream primer was: 5'- TCCTCTTACCGGTGGACTGG -3' (bases: 331 - 350), designed to generate a PCR product of 259 bp. The specificity of the primers was verified through Gene-Bank.

The PCR fragment amplified with PIN- specific primers was subcloned into a pCR 4-TOPO plasmid (Invitrogen, Carlsbad, CA) and sequenced in the University of Ottawa DNA sequencing facility.

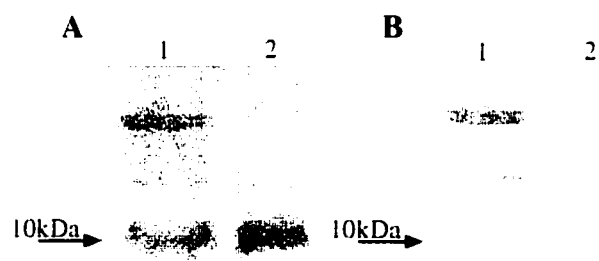
### **Immunohistochemistry**

Animals were anaesthetized with an intraperitoneal injection of 100 mg/kg sodium pentobarbital and the kidneys were removed and immersed in Zamboni's fixative (2% paraformaldehyde, 15% picric acid in 0.1 M phosphate buffer, pH 7.3) at 4° C overnight. Tissue was then rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 10% sucrose and incubated overnight at 4° C. The kidneys were embedded in paraffin and 5  $\mu$ m sections were cut with a microtome (Jung RM 2035) and mounted onto superfrost microscope slides. The kidney slices were deparaffinized by incubation in xylene for 5 min at room temperature, then transferred into fresh xylene for another 5 min. The slices were rehydrated by sequential incubation in 100%, 95% and 70% ethanol, for 5 min each at room temperature, followed by incubation in water for 2 min. The kidney slices were rinsed with PBS and endogenous peroxidase activity was blocked by incubation in a solution of 0.75% H<sub>2</sub>O<sub>2</sub> in methanol for 1 hr at room temperature. The sections were then permeabilized with 0.3% Triton X-100 in PBS for 15 min at room temperature. Slides were then incubated in blocking buffer consisting of PBS supplemented with 5% normal donkey serum (Sigma-Aldrich, Oakville, Ont., Canada) for 1 hr at room temperature. A goat polyclonal PIN antibody (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS supplemented with 2.5% donkey serum was added to the slides and incubated for 18 hrs at 4° C. To ensure specificity of staining with this antibody, additional slices were incubated in the presence of primary antibody that had been previously neutralized with the immunizing antigen (Santa Cruz Biotechnology), or in the presence of normal goat serum. In some experiments slides were incubated with 1:200 dilution of rabbit polyclonal antibody to

nNOS (Santa Cruz Biotechnology) for 18 hrs at 4° C. The slides were then washed 3 times for 5 min each in PBS and incubated with donkey anti-goat (for PIN) or anti-rabbit (for nNOS) biotinylated whole antibody (1:100 dilution, Amersham, Oakville, Ont., Canada) for 1 hr at room temperature. Following 3 washes with PBS, the slides were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature followed by incubation with 1:50 dilution of streptavidin-horseradish peroxidase (Amersham) in PBS for 1 hr at room temperature. The slides were washed and incubated with 3,3'-diaminobenzidine chromogen solution (DAB, BioGenex, San Ramon, CA) for 10 min. All slides were incubated in hematoxylin (Sigma), water, 95% and 100% ethanol and xylene. The slides were covered with Permount (Fisher Scientific, Nepean, Ont., Canada) and viewed with a Zeiss Axioplan microscope.

The specificity of the polyclonal PIN antibody utilized in immunohistochemistry studies was established by Western blotting (Figure 5.1). The antibody detected the 10 kDa PIN in both rat IMCD lysates and in rat brain lysate (positive control) (Figure 5.1A). In addition, in IMCD lysates the antibody detected three other proteins at approximately 18, 30, and 32 kDa. When the PIN antibody was preabsorbed with excess immunizing peptide, the signal for the 10 kDa PIN protein disappeared, but not the signals for the unidentified other three proteins (Figure 5.1B).

**Figure 5.1. Western blot demonstrating specificity of anti-PIN antibody.** Representative Western blot depicting PIN immunoreactivity with the polyclonal anti-PIN antibody utilized in immunohistochemical studies. Lane 1: 60 µg of IMCD lysate and lane 2: rat brain lysate as positive control. Incubation with the anti-PIN antibody results in the detection of 10 kDa PIN in lanes 1 and 2, and of three other proteins at 18, 30 and 32 kDa in lane 1 (A). Preabsorption of anti-PIN antibody with immunizing peptide results in selective blockade of PIN protein immunoreactivity (B).



### **Western blotting**

Cortices and inner medullae from sham and 5/6 Nx rats were homogenized with a cell disrupter in boiling lysis buffer (1% sodium dodecyl sulphate (SDS), 10 mM Tris-HCl, pH 7.4). Western blot analysis was performed as previously described (Roczniak et al., 1999). Briefly, the lysate was boiled for 10 min, followed by centrifugation at 12,000 x g for 2 min to remove insoluble debris. Protein concentrations in the supernatant were determined by the Bradford method (Bio-Rad, Montreal, Que., Canada) using bovine serum albumin (BSA, Sigma) as standard. Tissue lysates (20 µg for inner medulla, and 40 µg for cortex) were separated on 7.5% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% skimmed milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBS-T) and 0.01% sodium azide, for 1 hr at room temperature. The membranes were then incubated for 18 hrs at 4° C with 1:250 dilution of mouse monoclonal antibody to PIN (Transduction Laboratories, Lexington, KY), followed by incubation with 1:2000 dilution of anti-mouse secondary antibody conjugated to horseradish peroxidase (Amersham). A monoclonal antibody to PIN was used for Western blotting, since the polyclonal antibody used in the immunohistochemical experiments had lower sensitivity under the experimental conditions. Primary antibodies were diluted in TBS-T supplemented with 5% skimmed milk and 0.01% sodium azide, whereas the secondary antibodies were diluted in TBS-T supplemented with 2% milk. Proteins were detected by enhanced chemiluminescence (ECL, Amersham) on Hyperfilm (Amersham), according to the manufacturer's instructions. Prestained standards were used as molecular weight markers (Bio-Rad), and rat brain protein (4 µg, Transduction Laboratories) was used as a positive control for PIN. To control for protein loading, all membranes were stripped and probed with a monoclonal anti-β-actin antibody (mouse ascites fluid, Sigma), which recognized the β-actin protein at approximately 45 kDa. Signals on Western blots were quantified by densitometry and corrected for the β-actin signal, using an image analysis software program (NIH Image 1.47).

### **Statistics**

Results are presented as means ± SEM. Data were analyzed by the unpaired Student T- test. A value of  $p < 0.05$  was considered as significant.

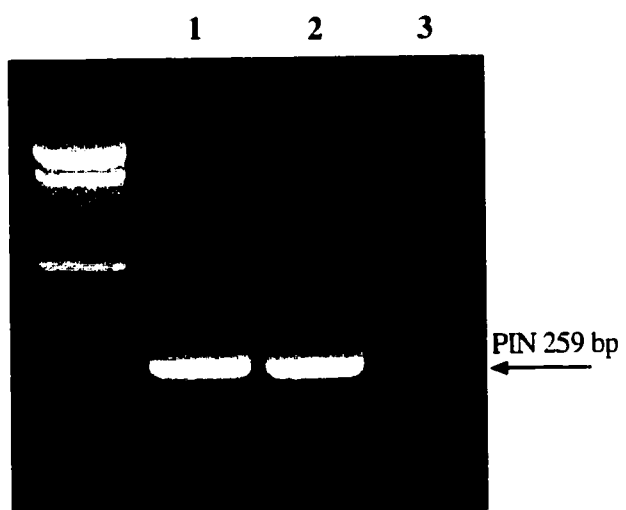
## RESULTS

### Localization of PIN

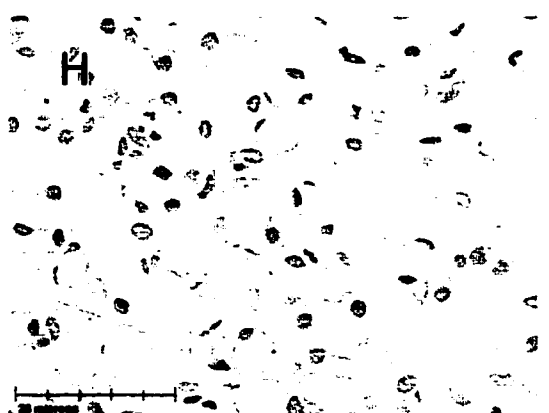
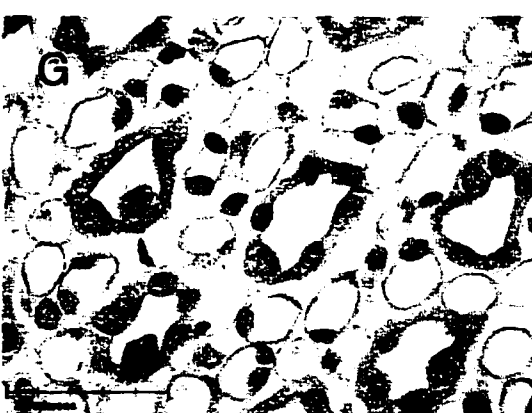
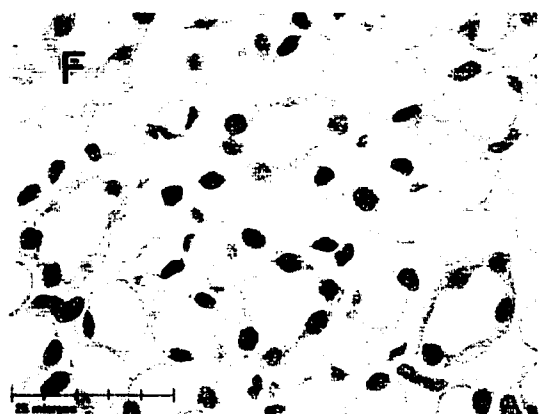
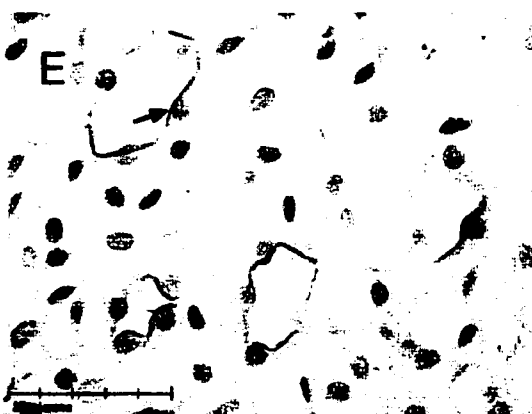
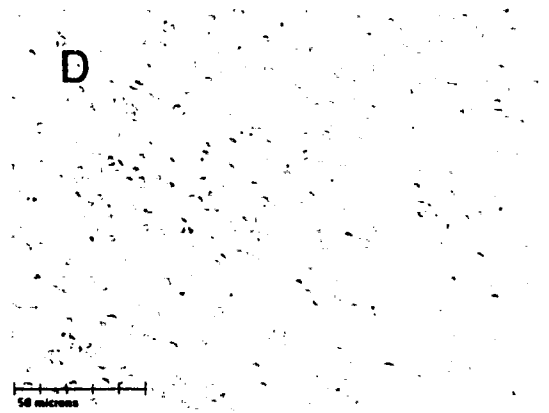
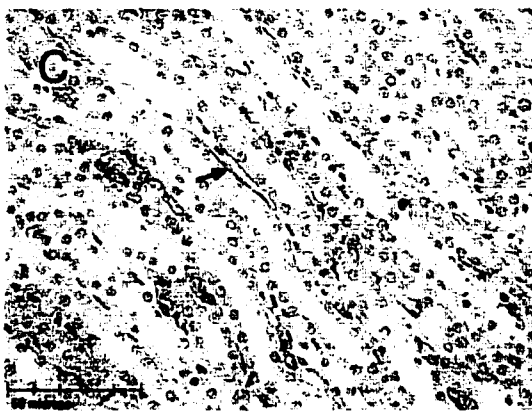
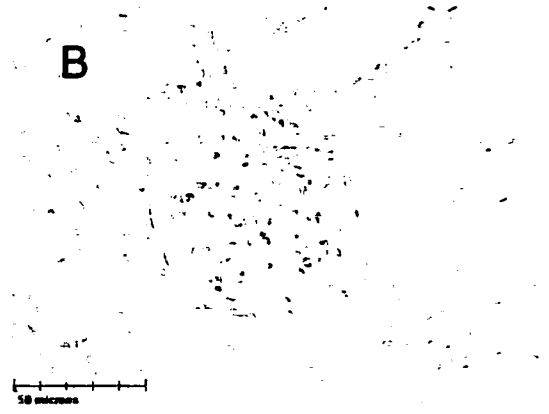
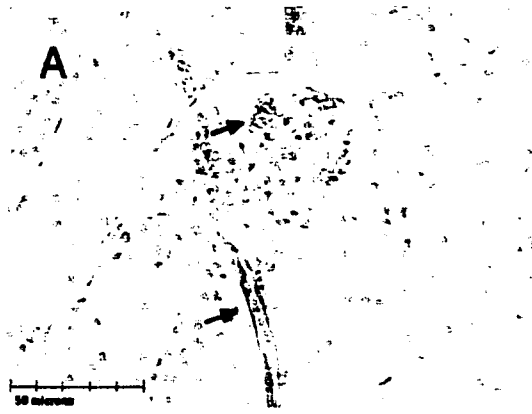
Using RT-PCR, mRNA expression for PIN was readily detected in both cortex and inner medulla of the rat kidney (Figure 5.2). By DNA sequencing, the nucleotide sequence of the PIN PCR product was identical to the published sequence for rat PIN (bases 91 to 350) (Jaffrey and Snyder, 1996). In order to localize PIN expression in the kidney we used immunohistochemistry. In the cortex, staining for PIN was observed in endothelial cells of the glomeruli and the afferent arterioles (Figure 5.3A), whereas in the outer medulla, endothelial cells of the vasa rectae stained for the presence of PIN (Figure 5.3C). In the inner medulla, staining for PIN was observed on the apical surface of the IMCD (Figure 5.3E). IMCD cells also demonstrate diffuse cytoplasmic staining for nNOS by immunohistochemistry (Figure 5.3G), consistent with our previous studies (Roczniak et al., 1999; Roczniak et al., 1998), and with recent work demonstrating relatively high levels of NOS activity in this segment (Wu et al., 1999). The specificity of staining was verified by incubating kidney slices with PIN antibody which had been previously neutralized with immunizing antigen, which resulted in complete loss of labeling in cortex, outer medulla and inner medulla (Figure 5.3B, D, and F). In addition, incubation with an identical dilution of nonimmune serum from goat resulted in absence of PIN staining of kidney slices, further demonstrating specificity of staining with the PIN antibody.

Kidney slices obtained from rats that underwent 5/6 nephrectomy were also examined for PIN distribution. The localization of PIN was unchanged compared to sham rats, with apical staining of IMCD, and staining of endothelial cells of the vasa rectae, afferent arterioles and glomeruli.

**Figure 5.2. RT-PCR for PIN in the kidney.** Representative ethidium bromide-stained agarose gel of cortical (lane 1) and inner medullary (lane 2) cDNA product (259 bp). Lane 3 depicts negative control obtained by omitting reverse - transcriptase.



**Figure 5.3. Immunolocalization of PIN.** Immunohistochemical micrographs of cortex (**A**), outer medulla (**C**), and inner medulla (**E**) of rat kidney labeled with polyclonal PIN antibody (arrow). In (**A**), upper arrow points to endothelial cells within a glomerulus; lower arrow indicates endothelial cells of afferent arteriole. Incubation of kidney sections with anti-PIN antibody preabsorbed with immunizing antigen demonstrates lack of staining in cortex, outer medulla, and inner medulla (**B, D, and F, respectively**). Micrograph of inner medulla labeled with a polyclonal antibody to nNOS is shown in (**G**), and depicts diffuse cytoplasmic staining of IMCD cells. To demonstrate antibody specificity, incubation of kidney sections with nNOS antibody preabsorbed with immunizing antigen demonstrates lack of inner medullary staining (**H**).



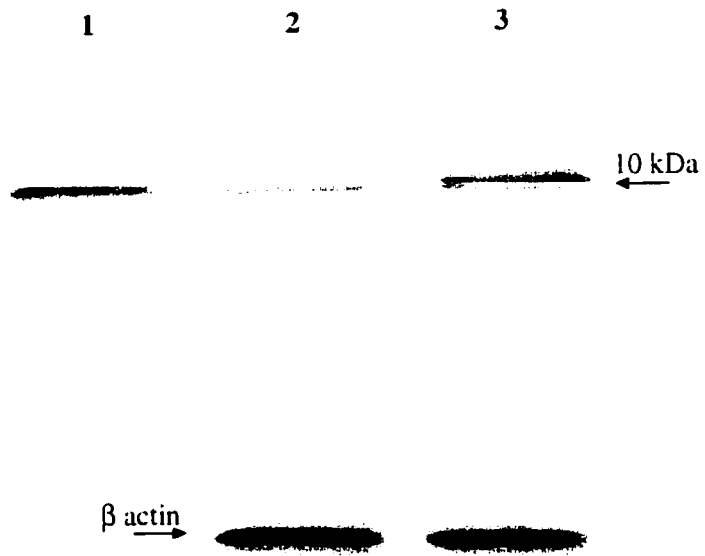
### **Effect of 5/6 Nx on expression of PIN in the inner medulla**

We have previously demonstrated that nNOS mRNA and protein expression is significantly downregulated in the cortex and inner medulla of 5/6 Nx rats (Roczniak et al., 1999). Accordingly, we studied the effect of 5/6 Nx on the expression of PIN, by Western blotting. Fourteen days after 5/6 Nx, PIN expression was significantly upregulated in the inner medulla (Figure 5.4: sham:  $0.18 \pm 0.07$  vs 5/6 Nx:  $0.58 \pm 0.13$  arbitrary units,  $p < 0.02$ ,  $n = 6$ ). Although by immunohistochemistry we were able to detect expression of PIN in glomerular and afferent arteriolar endothelial cells, Western blotting on proteins (40  $\mu$ g) extracted from the cortex revealed only a very faint band that did not permit adequate quantification by densitometry.

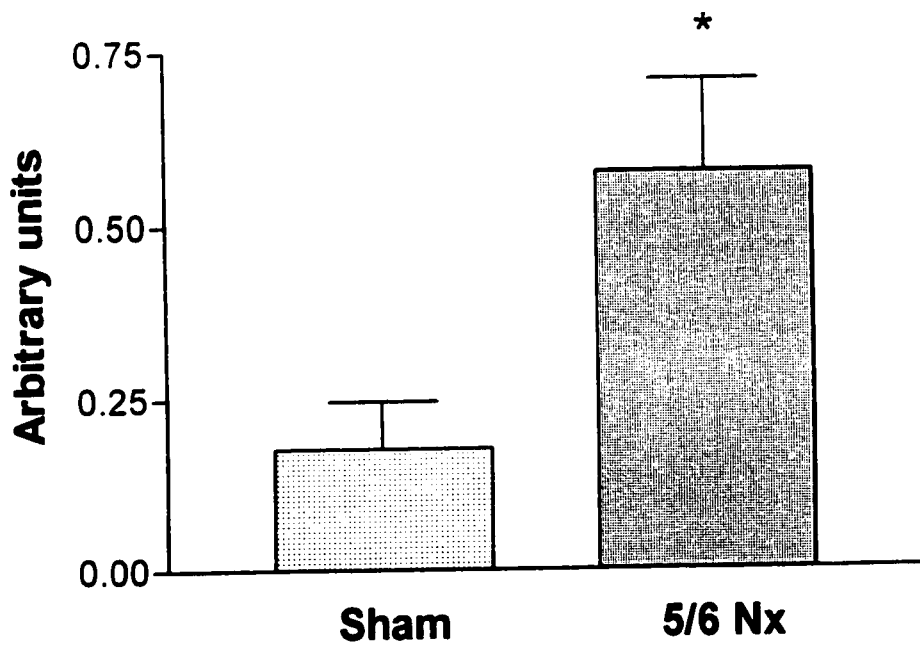
In all experiments, to control for possible variations in protein loading, membranes were stripped and probed for  $\beta$ -actin. There were no differences in  $\beta$ -actin protein abundance between samples derived from either sham or 5/6 Nx rats.

**Figure 5.4. Effect of 5/6 Nx on inner medullary expression of PIN.** (A) Representative Western blot of PIN expression in the inner medulla of sham and 5/6 Nx rats. Lane 1: rat brain lysate (positive control). Lane 2: 20  $\mu$ g total protein from sham rat. Lane 3: 20  $\mu$ g total protein from 5/6 Nx rat. Corresponding bands for  $\beta$ -actin are depicted, demonstrating equal loading of proteins. (B) Bar graph depicting densitometric quantification of Western blot signals for PIN in the inner medulla. Results are means  $\pm$  SEM (n=6), \*p< 0.02.

**A**



**B**



## DISCUSSION

A gene encoding PIN has recently been cloned from rabbit, and consists of three exons and two introns, spanning approximately 2.3 kb of genomic DNA (Jeong et al., 1998). Northern and Western blot analyses revealed that PIN is expressed in the testes, brain and respiratory muscles of humans, rats, and mice (Greenwood et al., 1997; Guo et al., 1999). PIN mRNA has also been detected by Northern blot analysis of whole human kidney RNA (Dick et al., 1996), but no studies have localized PIN within the kidney. In the present study we detected PIN immunoreactivity in endothelial cells of the glomeruli, afferent arterioles and vasa rectae. In the inner medulla, intense staining of the apical surface of IMCD cells, the main site of nNOS expression in the kidney (Roczniak et al., 1999; Roczniak et al., 1998), was observed. The expression of both PIN and nNOS in the IMCD suggests that nNOS activity, and hence NO production, may be tightly regulated by PIN. Although the macula densa expresses nNOS protein (Bachmann et al., 1995), it lacked immunoreactivity for PIN, suggesting that nNOS activity in the macula densa may be regulated in a PIN-independent fashion. While nNOS mRNA has been detected in the vasa recta (Terada et al., 1992), the afferent arteriole does not express significant levels of nNOS protein (Bachmann et al., 1995). Similarly, although nNOS has been detected in glomerular epithelial cells (Bachmann et al., 1995), it has not been detected in glomerular endothelial cells (Bachmann et al., 1995). The significance of the disparity in the distribution of nNOS and PIN in the kidney is unclear, but suggests that PIN may have other functions in addition to regulation of nNOS activity.

The cDNA for PIN, otherwise termed light chain dynein, was simultaneously cloned by two separate groups (Dick et al., 1996; Jaffrey and Snyder, 1996). One group identified this novel protein as an inhibitor of nNOS (Jaffrey and Snyder, 1996) whereas the other identified it as a light chain component of the dynein complex (Dick et al., 1996). Dyneins are highly complex molecular microtubule-based motors responsible for translocation of membranous vesicles, nuclear migration and movement of organelles (Hirokawa et al., 1998; Nagano et al., 1998). Cytoplasmic dynein is a multimeric protein consisting of two heavy chains, two or three intermediate chains and varying numbers of light chains (Hamm-Alvarez and Sheetz, 1998). The two heavy chains contain regions involved in ATP hydrolysis whereas the intermediate chains are thought to target dynein within the cell and may link the enzyme to the surface of membranous

organelles, kinetochores and vesicles (Hamm-Alvarez and Sheetz, 1998). The role of PIN or of any other light chain dynein in mammalian cells is unknown, but in *Drosophila*, partial loss-of-function mutations in light chain dynein are associated with morphogenic defects in bristle and wing development (Dick et al., 1996). In addition, diverse proteins have been found to interact and associate with PIN, including flagellar dynein (King and Patel-King, 1995), cytosolic dynein (King et al., 1996), I $\kappa$ B $\alpha$  (Crepieux et al., 1997), and nNOS (Fan et al., 1998; Jaffrey and Snyder, 1996). For example, in skeletal muscle PIN is found associated with the dynein complex as well as in the cytoplasm, suggesting it performs different functions (Guo et al., 1999). In Madin-Darby canine kidney (MDCK) cells, dynein is one of the microtubule motors involved in the apical transport of vesicles, suggesting its involvement in the maintenance of a polarized epithelium (Lafont et al., 1994). Dynein may also be involved in the transport of apical water and ion channels in the renal epithelia. Indeed, in IMCD cells, the intermediate dynein chain is associated with aquaporin-2 containing vesicles (Marple et al., 1998). The role of PIN in these processes is unknown, and to our knowledge, the present study is the first to determine its localization in the kidney. Our data, demonstrating localization of PIN in IMCD cells, suggest as one possibility that PIN may be involved in regulation of vesicular transport, and in regulation of nNOS activity in this segment.

In rats with renal mass reduction, both renal and systemic NO synthesis are reduced (Aiello et al., 1997). Administration of L-arginine to 5/6 Nx rats, reduced proteinuria, normalized creatinine clearance and increased fractional excretion of sodium, suggesting that NO is renoprotective in this model (Ashab et al., 1995). In a recent study, we showed that nNOS mRNA and protein expression is significantly downregulated in the cortex and inner medulla of 5/6 Nx rats (Roczniak et al., 1999). Furthermore, Vaziri et al. demonstrated that expression of intrarenal eNOS and iNOS is downregulated in this model of CRF (Vaziri et al., 1998). The decrease in NO production in CRF may also be due to an increase in the abundance of circulating endogenous inhibitors of NOS. The synthesis of NO can be inhibited by analogues of arginine, some of which are present in human plasma and are excreted in the urine (Vallance et al., 1992). Indeed, Vallance et al. showed that in patients with end-stage renal disease, the circulating concentration of the endogenous NOS inhibitor N<sup>G</sup>, N<sup>G</sup>-dimethyl arginine (ADMA) is significantly increased, to levels that can inhibit NOS activity (Vallance et al., 1992). In addition, urinary ADMA excretion significantly correlates with mean arterial pressure in Dahl salt-sensitive rats fed a high salt diet,

suggesting that ADMA may contribute to inhibition of NO synthesis and development of high blood pressure in this rat model of hypertension (Matsuoka et al., 1997). Our results indicate that PIN expression in the inner medulla was significantly upregulated in 5/6 Nx, suggesting that PIN could contribute to a decrease in NO synthesis in the inner medulla, accompanying CRF. Furthermore, it is possible that decreased NO production could contribute to long-term elevation of blood pressure, since inhibition of inner medullary nNOS expression has been shown to induce salt-sensitive hypertension in rats (Mattson and Bellehumeur, 1996).

In summary, this study shows that the endogenous inhibitor of nNOS, PIN, is expressed in the endothelial cells of the glomeruli, afferent arterioles and vasa rectae. In the inner medulla PIN is expressed in the IMCD, a site of high nNOS expression in the kidney. In 5/6 Nx rats, upregulation of PIN expression may contribute to salt retention and increased blood pressure, via inhibition of local NO synthesis.

## CHAPTER 6- GENERAL DISCUSSION

The goal of our research was to study the role and regulation of NO synthesis in the kidney. Our studies have shown that: 1) NO inhibits one of the major Na<sup>+</sup> transporters in the kidney, namely the NHE3 transporter in the PT, which is consistent with the role of NO as a natriuretic factor. 2) Neuronal NOS, one of the enzymes responsible for NO generation, is expressed at high levels in the IMCD, the renal segment responsible for the final adjustments in Na<sup>+</sup> and water reabsorption. In the IMCD, nNOS protein is distributed over the cytoplasm and the nucleus of the cells. In addition, we have localized PIN, an inhibitor of nNOS, in the endothelial cells of the glomerulus, afferent arteriole and vasa recta and in the IMCD. 3) High dietary salt intake differentially regulates nNOS expression in the cortex and the IMCD. In the cortex nNOS mRNA and protein expression are downregulated by high salt, whereas in the IMCD the protein levels for nNOS are upregulated. 4) In 5/6 Nx rats, nNOS mRNA and protein expression are significantly downregulated in the cortex and inner medulla. Furthermore, PIN levels in the inner medulla of 5/6 Nx rats are also significantly upregulated and may contribute to the overall low NO generation during CRF. 5) Administration of losartan, an AT<sub>1</sub> receptor blocker, did not reverse the inhibitory effect of 5/6 Nx on nNOS expression but significantly decreased nNOS mRNA and protein levels in the cortex and inner medulla of sham rats. This suggests that Ang II stimulates nNOS expression in the normal kidney.

### 6.1 Effect of NO on Apical NHE3 in the PT

The kidney is very sensitive to inhibition of NO synthesis. Administration of NOS inhibitors at concentrations that do not alter glomerular and systemic hemodynamics, decreases Na<sup>+</sup> excretion in rats (Deng et al., 1994; Deng et al., 1995; Shultz and Tolins, 1993; Tolins and Shultz, 1994), dogs (Salazar et al., 1993) and humans (Bech et al., 1996). Furthermore, administration of a bolus dose of NOS inhibitor to humans is accompanied by a decrease in Na<sup>+</sup> and fractional lithium excretion, suggesting that NO inhibits Na<sup>+</sup> reabsorption in the PT (Bech et al., 1996). In the PT, 65% of filtered Na<sup>+</sup> is reabsorbed mostly by the

action of the apical NHE3 exchanger (Schnermann and Sayegh, 1998). We have performed our initial set of experiments in order to determine whether NO regulates NHE3-mediated  $\text{Na}^+$  uptake in the PT.

Our study shows that two NO donors, SNP and SNAP, significantly reduce the NHE3-mediated  $\text{Na}^+$  uptake in the RPTC. The effect of NO on NHE3 is dependent on cGMP generation by the PT since incubation in the presence of a membrane permeable analogue of cGMP also decreases NHE3-mediated  $\text{Na}^+$  uptake. In addition, inhibition of sGC with a specific inhibitor, decreases cGMP generation in response to NO donors and reverses NO-induced inhibition of NHE3-mediated  $\text{Na}^+$  uptake.

Extensive evidence shows that NO directly inhibits  $\text{Na}^+$  transport in various nephron segments. In the PT, in addition to inhibition of NHE3, NO also inhibits the activity of the  $\text{Na}^+/\text{K}^+$ -ATPase (Guzman et al., 1995; Liang and Knox, 1999). We performed our studies in the presence of an inhibitor of the  $\text{Na}^+/\text{K}^+$ -ATPase activity. Therefore, the inhibition of NHE3-mediated  $\text{Na}^+$  uptake by NO is not secondary to inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase by NO. Hence, we can conclude that NO inhibits  $\text{Na}^+$  reabsorption by acting on the two major  $\text{Na}^+$  transporters in the PT. In the TAL, endogenously produced NO inhibits  $\text{Na}^+/\text{K}^+$ -ATPase  $\alpha$ 1-subunit gene transcription and consequently, sodium pump activity (Kone and Higham, 1999). In the CCD, endogenously produced NO inhibits amiloride-sensitive  $\text{Na}^+$  uptake (Stoos et al., 1992; Stoos et al., 1994; Stoos et al., 1995) and AVP-stimulated water permeability (Garcia et al., 1996; Garcia et al., 1996).

A recent study contradicted the results obtained in PT cells. In microperfused PT tubules, infusion of L-NAME into the lumen of the tubule decreases  $\text{Na}^+$  transport suggesting that NO increases  $\text{Na}^+$  reabsorption in the PT (Wang, 1997). Furthermore, PT perfusion with low NO concentrations stimulates NHE3 exchanger activity, and inhibits NHE3 activity at high concentrations (Wang, 1997). The author concluded that NO has a dual effect on PT  $\text{Na}^+$  reabsorption: a stimulatory effect at low concentrations, and an inhibitory one at high concentrations. Although interesting, this study remains at odds with both *in vitro* and *in vivo* experiments performed to date and warrants further investigation.

The identity of the isoform of NOS involved in the inhibition of renal  $\text{Na}^+$  reabsorption remains unknown. The iNOS mRNA is expressed in the PT, TAL and IMCD at basal levels (Ahn et al., 1994; Mohaupt et al., 1994) and its expression is augmented by cytokines (Ahn et al., 1994; Markewitz et al., 1993). Indeed, cytokine-induced iNOS expression causes inhibition of  $\text{Na}^+/\text{K}^+$  ATPase activity in mouse

PT (Guzman et al., 1995) and TAL cells (Kone and Higham, 1999), which leads to a net decrease in  $\text{Na}^+$  reabsorption in both segments. It remains to be shown however, that iNOS in the absence of immune stimulation regulates renal  $\text{Na}^+$  reabsorption. Furthermore, the ecNOS isoform may be expressed in PT cells and in the peritubular capillaries that surround the PT, and may tonically modulate  $\text{Na}^+$  transport. However, the studies to that effect are inconclusive. One study showed that NO released from the endothelium of the peritubular capillaries stimulates the activity of NHE3 (Amorena and Castro, 1997), whereas the other using a coculture of PT and endothelial cells showed that NO inhibits  $\text{Na}^+$  transport in the PT (Linas and Repine, 1999). The nNOS isoform is not expressed in either the PT or TAL (Bachmann et al., 1995; Rocznik et al., 1999) but is highly expressed in the IMCD (Rocznik et al., 1998) and at lower levels in the CCD (Wang et al., 1998) and may be involved in the regulation of  $\text{Na}^+$  transport in these segments, but no studies addressing this have been performed.

Our study also shows that NO-mediated inhibition of NHE3 in the PT is dependent on cGMP generation. The targets for cGMP in PT cells are presently unknown but two possibilities may be considered: 1) inhibition of PDE3 activity and 2) stimulation of cGK activity (Lane and Gross, 1999). Binding of cGMP to PDE3 inhibits its activity and accordingly increases cAMP levels which could activate PKA (Schmidt et al., 1993). PKA is one of the most potent inhibitors of NHE3 in the PT (Kurashima et al., 1997). The C-terminal of the NHE3 transporter is cytoplasmic and binds to two regulatory factors, NHERF and E3KARP (Lamprecht et al., 1998). Stimulation of PKA activity results in the NHERF and E3KARP-mediated phosphorylation of the C-terminal of NHE3 and inhibition of its activity (Zizak et al., 1999). To test the possibility that the NO-mediated decrease in  $\text{Na}^+$  uptake by NHE3 is due to PKA activation we measured PKA activity. However, measurement of PKA activity in PT cells pretreated with an NO donor revealed that the activity of the kinase remained unchanged.

Regulation of NHE3 by cGK has not been described, but putative sites for phosphorylation by kinases are found in the C-terminal of NHE3 (Wakabayashi et al., 1997). Two isoforms of cGK, namely cGKI and cGKII, have been identified. Although cGKI is not expressed in the PT, high levels of cGKII have been detected in this renal segment (Gambaryan et al., 1996).

In conclusion, our study shows that NO inhibits NHE3-mediated  $\text{Na}^+$  uptake in the PT and that this effect is mediated by cGMP generation. We suggest that basal renal NO synthesis plays an important

role in determining extracellular volume composition and regulation of blood pressure. Hence, deficient NO synthesis in the kidney could contribute to impaired  $\text{Na}^+$  excretion and development of hypertension.

## 6.2 Expression of nNOS and PIN in the kidney

Although both the renal cortex and medulla produce NO, studies demonstrated that the latter is the principal site for basal NO synthesis in the kidney (Zou and Cowley, 1997). Furthermore, a recent study performed on microdissected renal segments demonstrated that NOS activity is highest in the IMCD (Wu et al., 1999). These observations are particularly intriguing due to the well documented importance of the renal medulla in the mediation of pressure natriuresis (Cowley, 1997). We performed experiments to determine whether iNOS and nNOS proteins are expressed in the IMCD.

Our results show that in the cortex nNOS protein is predominantly localized in the macula densa, which is in agreement with previous reports by Bachmann et al (Bachmann et al., 1995). In addition, our study shows for the first time, that high levels of nNOS are expressed in the IMCD. Although we have detected iNOS mRNA in IMCD by RT-PCR, we did not detect iNOS protein by western blotting. This is probably due to the lack of specificity of the commercially available iNOS antibodies, since Tojo et al., using their own antibody detected iNOS immunoreactivity in the intercalated cells of the CCD (Tojo et al., 1994).

The distribution of nNOS in the kidney differs markedly from that of either ecNOS or iNOS. The ecNOS protein is predominantly expressed in the vascular endothelial cells and may be expressed in the PT (Kone, 1999; Ujiiie et al., 1994). The iNOS mRNA expression is widespread in comparison to both nNOS and ecNOS. Inducible NOS transcripts are found in the PT, TAL, CCD, and IMCD, the interstitial cells, glomerular mesangial cells and the vascular smooth muscle cells (VSM) cells (Mohaupt et al., 1994; Mohaupt et al., 1995). These studies demonstrate that the kidney has a great capacity for NO generation. Furthermore, the differential localization of the three isoforms of NOS in the kidney suggests that these isoforms may perform separate functions.

Using immunogold labeling we showed that nNOS is evenly distributed over the cytoplasm and nucleus of the IMCD cells. The role of nuclear nNOS expression is unknown and merits further investigation. However, it should be noted that nuclear capacity to produce NO is consistent with the

postulated role of NO in the regulation of transcription factor activity and gene expression (Idriss et al., 1999; Klatt et al., 1999; Nunoshiba et al., 1993; von Knethen et al., 1999).

The activity of nNOS is tightly regulated by protein-protein interactions and intracellular  $\text{Ca}^{2+}$  concentration (Christopherson and Bredt, 1997). Recently, a protein inhibitor of nNOS, PIN, has been isolated from rat brain (Jaffrey and Snyder, 1996) and shown to bind to a 17 amino acid sequence localized in the N-terminal of nNOS (Fan et al., 1998). The binding of PIN to nNOS destabilizes the dimeric structure of nNOS and inhibits its activity (Fan et al., 1998). Since nNOS is expressed in the kidney and may be involved in a number of important functions, including regulation of renin secretion and mediation of pressure natriuresis (Kone, 1997), we determined whether PIN is also expressed in the kidney. Our data show that PIN is principally expressed in the IMCD, the site of highest nNOS expression in the kidney, and that it is associated with the apical membrane. In the cortex, we detected PIN immunoreactivity in the endothelial cells of the glomerular capillaries and afferent arteriole, whereas in the outer medulla PIN was detected in the endothelial cells of the vasa recta.

Our observations suggest that at least in the IMCD nNOS activity, and thus NO synthesis, may be regulated by PIN. Furthermore, our studies suggest that different pools of nNOS might exist in the IMCD. One pool of nNOS localized at the apical membrane may interact with PIN, whereas a second pool of nNOS distributed over the cytoplasm and basolateral membrane does not colocalize with PIN expression and may be regulated by other as yet unidentified proteins. It should be noted however, that PIN has also been cloned by another group and identified as a light chain component of the cellular dynein complex (Dick et al., 1996), suggesting that PIN may perform functions that are unrelated to regulation of nNOS activity in the IMCD. Indeed, cellular dynein is involved in the transport of vesicles to the apical membrane of collecting duct derived MDCK cells (Hamm-Alvarez and Sheetz, 1998; Lafont et al., 1994) and in the IMCD cells the intermediate dynein chain is associated with aquaporin-2 vesicles (Marple et al., 1998).

The significance of PIN in endothelial cells of the cortex and outer medulla is at the moment difficult to explain given that nNOS is not expressed in these cells. One explanation however, could be that PIN regulates ecNOS activity. Indeed, PIN inhibits the activity of purified ecNOS (Hemmens et al., 1998),

yet the I7 amino acid binding sequence for PIN which is present in nNOS, is absent in ecNOS (Fan et al., 1998). Further studies are required to clarify the role of PIN in endothelial cells.

In summary, our study shows that the two principle sites of nNOS expression in the kidney are the macula densa in the cortex and the IMCD cells in the medulla. Furthermore, PIN, an endogenous inhibitor of nNOS, is highly expressed at the apical membrane of IMCD where it might regulate nNOS activity. Since we did not detect PIN immunoreactivity in the macula densa, our study suggests that this segment might utilize a different mechanism for regulation of nNOS activity. The role of PIN in the endothelial cells of the glomerular arterioles, afferent arteriole and vasa recta remains unknown.

### **6.3 Effect of High Dietary Salt on nNOS Expression in the Cortex and IMCD**

NO regulates renal Na<sup>+</sup> excretion by inhibiting tubular Na<sup>+</sup> reabsorption and by regulating medullary hemodynamics. We showed that NO inhibits NHE3-mediated Na<sup>+</sup> uptake, whereas other studies demonstrated that NO blocks Na<sup>+</sup> reabsorption in the TAL (Kone and Higham, 1999) and CCD (Stoos et al., 1995). In the medulla, stimulation of NO synthesis increases renal medullary blood flow whereas inhibition of NO synthesis inhibits medullary blood flow (Mattson et al., 1997). The changes in the medullary blood flow in response to stimulation or inhibition of NO synthesis result in parallel changes in Na<sup>+</sup> and water excretion (Mattson et al., 1997). The IMCD in the medulla expresses high levels of both nNOS (Roczniak et al., 1999) and iNOS (Ahn et al., 1994; Mohaupt et al., 1994; Mohaupt et al., 1995). Inhibition of medullary nNOS activity by infusion of an antisense to nNOS decreases renal medullary blood flow and is associated with increased Na<sup>+</sup> retention in rats fed a high Na<sup>+</sup> diet (Mattson and Bellehumeur, 1996). Although inhibition of iNOS by systemic infusion of aminoguanidine did not affect medullary blood flow, it was associated with Na<sup>+</sup> retention and development of hypertension in rats fed a high Na<sup>+</sup> diet (Mattson et al., 1998). These studies suggest that both nNOS and iNOS may be involved in the regulation of Na<sup>+</sup> reabsorption in the kidney. Schultz and Tolins showed that plasma and urine content of nitrates/nitrites, the metabolic products of NO, are increased in animals fed a high salt diet (Shultz and Tolins, 1993) and suggested that an increase in NO production during high dietary Na<sup>+</sup> intake promotes vasodilatation and natriuresis and helps to maintain body Na<sup>+</sup> balance. The goal of our next study was to

determine whether high dietary salt intake regulates expression of nNOS and iNOS mRNA and protein in the IMCD.

Our results show that after 3 days of a high salt diet the nNOS mRNA and protein expression in the cortex are significantly downregulated. Decreased nNOS mRNA expression in response to a high salt diet may be due to either decreased transcription of nNOS gene or decreased nNOS mRNA stability. In contrast, our data shows that nNOS protein levels after 3 days of high salt diet, are upregulated in the IMCD despite the absence of an effect on mRNA expression, suggesting an increase in translation or stability of nNOS protein. Our study uncovered the very interesting observation that the expression of nNOS in the cortex and the IMCD is differentially regulated in response to the same stimuli. The differential regulation of nNOS expression in the cortex and the IMCD by salt may be due to transcription from cell-specific promoters. Indeed at least nine different 5'UTR, probably resulting from transcription from different promoters have been isolated and shown to be tissue specific (Wang et al., 1999). Accordingly, it is conceivable that nNOS transcription in the cortex and in the IMCD is driven by separate promoters. Furthermore, the 5'UTRs of nNOS mRNA contain cis RNA elements that modulate translational efficiency in response to changes in cellular phenotype (Wang et al., 1999). This suggests that translational efficiency of nNOS mRNA in the IMCD could be increased in response to a high salt-diet.

After 3 weeks of the high salt diet nNOS protein levels in the IMCD are no longer different from controls. Despite the absence of an effect on nNOS expression in the IMCD, urinary nitrate/nitrite excretion is still significantly elevated. An unexplored explanation for this is that nNOS activity is regulated by dietary Na<sup>+</sup> intake. Indeed, the activity of nNOS is tightly regulated by targeting of nNOS to different cellular compartments (Christopherson and Brecht, 1997), and by the interaction of nNOS with various proteins such as the endogenous inhibitor of nNOS, PIN (Jaffrey and Snyder, 1996). As we have previously demonstrated PIN is highly expressed in the IMCD but its function remains unknown. Thus, it is possible that chronic high salt intake alters the distribution of nNOS in the IMCD or its association with other cellular proteins, such as PIN. Another possibility, is the upregulation of either ecNOS or iNOS protein expression by high dietary salt. Indeed, Mattson et al., demonstrated that both ecNOS and iNOS protein levels are upregulated in the kidneys of rats fed a high salt diet for 3 weeks (Mattson and Higgins, 1996).

Since nNOS in the cortex and IMCD is differentially regulated by salt, our study suggests that nNOS may play different roles in these renal segments. In the cortex, the nNOS expressed in the macula densa is involved in the stimulation of renin secretion (Kurtz et al., 1998; Kurtz and Wagner, 1998). The changes in the macula densa nNOS mRNA in response to dietary salt, furosemide or in two-kidneys-one-clip hypertension vary in parallel with the changes in renin expression and secretion from the granular cells (Bosse et al., 1995; Schricker et al., 1996; Singh et al., 1996), supporting the role of NO in the regulation of renin secretion. Therefore, decreased expression of nNOS in the cortex during high salt intake is consistent with the postulated role of macula densa-derived NO in the stimulation of renin secretion.

In the IMCD an increase in NO synthesis during high salt intake may play a role in the induction of natriuresis. The role of nNOS in IMCD has never been directly investigated but there are a number of possibilities by which it could regulate  $\text{Na}^+$  excretion. Generation of NO in the IMCD and diffusion of NO to the surrounding vasa recta may result in increased vasodilatation and increased medullary blood flow (Mattson et al., 1997). Changes in medullary blood flow are associated with parallel changes in the  $\text{Na}^+$  and water excretion (Mattson et al., 1997). Therefore, increased blood flow to the papilla may increase the interstitial hydrostatic pressure which in turn may decrease  $\text{Na}^+$  uptake in the mTAL, OMCD, and IMCD (Cowley et al., 1992). The IMCD also reabsorbs a small portion of filtered  $\text{Na}^+$ , by the action of the apically expressed ENaC (Volk et al., 1995). The regulation of ENaC by NO has never been demonstrated but it is of interest that in the CCD, which also reabsorbs  $\text{Na}^+$  by the activity of ENaC, endogenous NO significantly inhibits amiloride sensitive  $\text{Na}^+$  uptake (Stoos et al., 1994; Stoos et al., 1995). In addition, exposure of IMCD cells to NO donors also decreased  $\text{Na}^+$  uptake, presumably by inhibiting the  $\text{Na}^+/\text{K}^+$  ATPase activity (Zeidel et al., 1986).

In summary, our studies show that dietary  $\text{Na}^+$  differentially regulates nNOS expression in the cortex and the IMCD. In the cortex, 3 days of high dietary  $\text{Na}^+$  decreases nNOS mRNA and protein expression whereas in the IMCD nNOS protein levels are upregulated without an effect on nNOS mRNA. These studies suggest that in the cortex nNOS may be involved in the regulation of renin secretion whereas in the IMCD nNOS may be involved in the regulation of medullary blood flow and of  $\text{Na}^+$  reabsorption by the IMCD.

## 6.4 Expression of nNOS and PIN in the Rat Model of CRF

Extensive renal mass reduction (RMR) in rats is a model of CRF. In these animals both GFR and RBF are significantly decreased despite an initial adaptive increase in *sn*GFR (Anderson et al., 1985). Rats with RMR develop systemic hypertension, severe proteinuria and structural changes in the kidney which include glomerulosclerosis (Anderson et al., 1985). These changes eventually lead to renal insufficiency (Anderson et al., 1985). Renal and systemic NO production in CRF is greatly decreased in patients with end-stage renal disease (Schmidt et al., 1999) and in rats with RMR (Aiello et al., 1997; Ashab et al., 1995) as evidenced by decreased levels of nitrites/nitrates in the plasma and urine. Administration of L-arginine alleviates the progression of CRF by increasing NO production, reducing glomerular scarring and proteinuria (Ashab et al., 1995). The goal of our study was to determine whether nNOS and PIN expression are altered in rats with 5/6 Nx.

Our study demonstrates that nNOS mRNA expression and protein levels in the cortex and inner medulla of 5/6 Nx rats are significantly reduced. Similarly, a progressive loss of immunoreactivity for iNOS in the glomeruli, PT and collecting ducts (Aiello et al., 1997; Vaziri et al., 1998) and of ecNOS protein levels (Vaziri et al., 1998) was demonstrated in rats with RMR. There is also evidence that circulating levels of endogenous inhibitors increase in renal failure (Vallance et al., 1992). For instance, ADMA is a potent nonselective NOS inhibitor and its plasma levels increase in patients with CRF by about 8-fold, which is sufficient to block NOS activity (Vallance et al., 1992). We have previously demonstrated that PIN is highly expressed in the IMCD, the site of nNOS expression, therefore we determined whether its expression is altered in rats with 5/6 Nx. Our results show that PIN is significantly upregulated in the inner medulla of 5/6 Nx rats. Taken together these studies suggest that the decrease in renal NO synthesis is at least in part mediated by the loss of nNOS, ecNOS and iNOS expression in the kidney and by the increase in the levels of endogenous inhibitors of NOS such as PIN.

There are a number of foreseeable consequences of reduced NO synthesis in the kidney. For instance, inhibition of cortical NO synthesis increases afferent arteriole resistance (Ito and Ren, 1993), decreases  $K_f$  (Zatz and de Nucci, 1991), and enhances the TGF response (Wilcox et al., 1992). In the medulla on the other hand, reduced NOS activity leads to reduced renal blood flow and  $\text{Na}^+$  retention (Mattson and Bellehumeur, 1996). Overall, reduced NO synthesis may lead to development of

hypertension and glomerulosclerosis. Indeed, in patients with end-stage renal disease, hypertension is prevalent (Rostand et al., 1991). Interestingly, oral supplementation of L-arginine in rats with RMR and hypertension improved blood pressure control, and renal function (Ashab et al., 1995), suggesting that reduced NO synthesis in CRF is partially responsible for the development of hypertension and progression of renal injury.

In summary, our results show that nNOS mRNA and protein expression are reduced in the cortex and inner medulla of 5/6 Nx rats. In addition, PIN is upregulated in the inner medulla of 5/6 Nx rats. These results suggest that downregulation of nNOS expression in the cortex and medulla and increased PIN expression may in part be responsible for reduced NO generation in CRF.

### **6.5 Effect of Ang II AT<sub>1</sub> Receptors on the Expression of nNOS in the Kidney**

The RAS has been implicated in the progression of renal injury in both experimental models and in various forms of renal disease (Rosenberg et al., 1994). The deleterious effects of Ang II may be due to an increase in glomerular capillary pressure (Anderson et al., 1985) or an upregulation of cytokines such as the platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (Johnson et al., 1992; Kagami et al., 1994). Blockade of the RAS system reduces proteinuria and blood pressure in rats with RMR (Ashab et al., 1995; Junaid et al., 1997), at least partly by stimulating NO synthesis (Ashab et al., 1995). The goal of our study was therefore, to determine whether blockade of Ang II AT<sub>1</sub> receptors could reverse the downregulation of nNOS expression in the cortex and inner medulla of 5/6 Nx rats.

Our study shows that losartan, an AT<sub>1</sub> blocker, did not reverse the inhibitory effect of 5/6 Nx on inner medullary nNOS expression. In contrast, losartan had a small but statistically significant effect on nNOS mRNA and protein expression in the cortex. However, since the effect is small we must conclude, that other factors in addition to Ang II are responsible for nNOS downregulation in 5/6 Nx rats. At the moment, the mechanism responsible for mediating nNOS downregulation in the cortex and inner medulla of 5/6 Nx rats is unknown. It should be noted however, that the parathyroid hormone (PTH) has been implicated in the downregulation of both iNOS and eNOS in the kidney and aorta of rats with chronic insufficiency (Vaziri et al., 1998). The effect of PTH on nNOS expression is unknown. Furthermore, excessive synthesis of PDGF (Floege et al., 1992), TGF- $\beta$  (Lee et al., 1995), and of a potent

vasoconstrictor, endothelin-1 (Orisio et al., 1993), have been reported in rats with RMR. However, the effect of these factors on nNOS expression has not been examined.

Our studies also show that in sham-operated rats treated with losartan, cortical and inner medullary expression of nNOS mRNA and protein significantly decreased, suggesting that Ang II tonically upregulates nNOS expression in the kidney by acting on AT<sub>1</sub> receptors. This is consistent with the study by Zou et al., who demonstrated that medullary infusion of Ang II results in increased NO production (Zou et al., 1998). There are at least two Ang II receptors in the kidney. The AT<sub>1</sub> is expressed on the apical and basolateral sides of renal tubules and in the vasculature and is responsible for Ang II-induced vasoconstriction and Na<sup>+</sup> reabsorption (Hall et al., 1999). Activation of AT<sub>1</sub> receptors has previously been shown to increase ecNOS gene transcription (Hennington et al., 1998) and inhibit iNOS expression in a PT-like cell line (Wolf et al., 1997). The Ang II AT<sub>2</sub> receptor is present at low levels in the adult kidney (Ozono et al., 1997), and its activation has been associated with the regulation of natriuresis (Lo et al., 1995). Activation of AT<sub>2</sub> receptors leads to generation of NO (Siragy and Carey, 1997), but the isoform of NOS that is regulated by this receptor is presently unknown. Hence, Ang II increases renal NO production by acting on both AT<sub>1</sub> and AT<sub>2</sub> receptors. Furthermore, Ang II increases expression of ecNOS and nNOS but not iNOS.

In summary, our study shows that Ang II is not the major factor responsible for the downregulation of cortical and inner medullary nNOS expression in 5/6 Nx rats. In addition, in normal rats, tonic stimulation of AT<sub>1</sub> receptors by Ang II results in upregulation of nNOS expression in the cortex and inner medulla. The Ang II-induced renal NO synthesis may be a protective action against Ang II-mediated intrarenal vasoconstriction and Na<sup>+</sup> reabsorption.

## **6.6 Summary**

The aim of our studies was to determine the role and regulation of NO synthesis in the kidney. Our results may be summarized as follows:

1) NO inhibits NHE3-mediated  $\text{Na}^+$  uptake in the PT in a cGMP dependent manner. Our study suggests that one of the possible mechanisms for induction of pressure natriuresis is a direct inhibition of PT  $\text{Na}^+$  uptake by NO.

2) The two major sites for nNOS expression in the kidney are the macula densa and the IMCD. In the IMCD, nNOS protein is localized in the cytosol and the nuclei. High dietary  $\text{Na}^+$  differentially regulates nNOS mRNA and protein expression in the cortex and IMCD. In the cortex, high dietary  $\text{Na}^+$  decreases nNOS mRNA expression and protein levels, whereas in the IMCD nNOS protein levels are upregulated independently of an effect on nNOS mRNA expression. Downregulation of nNOS in the cortex is consistent with the postulated role of nNOS in the stimulation of renin secretion, whereas upregulation of nNOS expression in the IMCD is consistent with the postulated role of nNOS in the regulation of medullary blood flow and  $\text{Na}^+$  excretion. The effect of high dietary  $\text{Na}^+$  on nNOS expression in the IMCD is transient, and returns to control levels after 3 weeks, suggesting that NO production may be regulated by a different mechanism during chronic high dietary  $\text{Na}^+$  intake.

3) CRF is characterized by low NO production. In 5/6 Nx rats both cortical and inner medullary nNOS expression is significantly downregulated, suggesting that a decrease in renal nNOS expression may contribute to reduced NO synthesis in CRF. Treatment with losartan did not reverse 5/6 Nx-induced downregulation of inner medullary nNOS expression but had a small but significant effect on cortical nNOS expression. Furthermore, in sham rats losartan induced a significant downregulation of cortical and inner medullary nNOS expression, suggesting that in normal rats Ang II tonically upregulates nNOS expression.

4) An endogenous inhibitor of nNOS, PIN, is expressed in the endothelial cells of the glomerular arterioles, the afferent arteriole and the vasa recta. In addition, PIN is expressed on the apical membrane of the IMCD, a site of high renal nNOS expression. In 5/6 Nx rats, inner medullary PIN expression is significantly upregulated, suggesting that it may be in part responsible for decreased renal NO synthesis in CRF.

## CHAPTER 7: REFERENCES

Abu-Soud, H. M., Feldman, P. L., Clark, P., and Stuehr, D. J. (1994). Electron transfer in the nitric-oxide synthases. Characterization of L- arginine analogs that block heme iron reduction. *J Biol Chem* 269, 32318-26.

Abu-Soud, H. M., Gachhui, R., Raushel, F. M., and Stuehr, D. J. (1997). The ferrous-dioxy complex of neuronal nitric oxide synthase. Divergent effects of L-arginine and tetrahydrobiopterin on its stability. *J Biol Chem* 272, 17349-53.

Abu-Soud, H. M., Yoho, L. L., and Stuehr, D. J. (1994). Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. *J Biol Chem* 269, 32047-50.

Ahn, K. Y., Mohaupt, M. G., Madsen, K. M., and Kone, B. C. (1994). In situ hybridization localization of mRNA encoding inducible nitric oxide synthase in rat kidney. *Am J Physiol* 267, F748-57.

Aiello, S., Noris, M., Todeschini, M., Zappella, S., Foglieni, C., Benigni, A., Corna, D., Zoja, C., Cavallotti, D., and Remuzzi, G. (1997). Renal and systemic nitric oxide synthesis in rats with renal mass reduction. *Kidney Int* 52, 171-81.

Alberola, A., Pinilla, J. M., Quesada, T., Romero, J. C., Salom, M. G., and Salazar, F. J. (1992). Role of nitric oxide in mediating renal response to volume expansion. *Hypertension* 19, 780-4.

Amlal, H., LeGoff, C., Vernimmen, C., Soleimani, M., Paillard, M., and Bichara, M. (1998). ANG II controls Na(+)-K+(NH4+)-2Cl- cotransport via 20-HETE and PKC in medullary thick ascending limb. *Am J Physiol* 274, C1047-56.

- Amorena, C., and Castro, A. F. (1997). Control of proximal tubule acidification by the endothelium of the peritubular capillaries. *Am J Physiol* 272, R691-4.
- Anderson, R. G. (1993). Caveolae: where incoming and outgoing messengers meet. *Proc Natl Acad Sci U S A* 90, 10909-13.
- Anderson, S., Meyer, T. W., and Brenner, B. M. (1985). The role of hemodynamic factors in the initiation and progression of renal disease. *J Urol* 133, 363-8.
- Anderson, S., Meyer, T. W., Rennke, H. G., and Brenner, B. M. (1985). Control of glomerular hypertension limits glomerular injury in rats with reduced renal mass. *J Clin Invest* 76, 612-9.
- Ashab, I., Peer, G., Blum, M., Wollman, Y., Chernihovsky, T., Hassner, A., Schwartz, D., Cabili, S., Silverberg, D., and Iaina, A. (1995). Oral administration of L-arginine and captopril in rats prevents chronic renal failure by nitric oxide production. *Kidney Int* 47, 1515-21.
- Bachmann, S., Bosse, H. M., and Mundel, P. (1995). Topography of nitric oxide synthesis by localizing constitutive NO synthases in mammalian kidney. *Am J Physiol* 268 (*Renal Fluid Electrolyte Physiol.* 37), F885-98.
- Baek, K. J., Thiel, B. A., Lucas, S., and Stuehr, D. J. (1993). Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. *J Biol Chem* 268, 21120-9.
- Baylis, C., Harton, P., and Engels, K. (1990). Endothelial derived relaxing factor controls renal hemodynamics in the normal rat kidney. *J Am Soc Nephrol* 1, 875-81.

- Baylis, C., Harvey, J., and Engels, K. (1994). Acute nitric oxide blockade amplifies the renal vasoconstrictor actions of angiotension II. *J Am Soc Nephrol* 5, 211-4.
- Baylis, C., Mitruka, B., and Deng, A. (1992). Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage. *J Clin Invest* 90, 278-81.
- Beavo, J. A. (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* 75, 725-48.
- Bech, J. N., Nielsen, C. B., Ivarsen, P., Jensen, K. T., and Pedersen, E. B. (1998). Dietary sodium affects systemic and renal hemodynamic response to NO inhibition in healthy humans. *Am J Physiol* 274, F914-23.
- Bech, J. N., Nielsen, C. B., and Pedersen, E. B. (1996). Effects of systemic NO synthesis inhibition on RPF, GFR, UNa, and vasoactive hormones in healthy humans. *Am J Physiol* 270, F845-51.
- Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 87, 1620-4.
- Beckman, J. S., and Koppenol, W. H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271, C1424-37.
- Beckmann, J. S., Ye, Y. Z., Anderson, P. G., Chen, J., Accavitti, M. A., Tarpey, M. M., and White, C. R. (1994). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe Seyler* 375, 81-8.

Beierwaltes, W. H. (1995). Selective neuronal nitric oxide synthase inhibition blocks furosemide-stimulated renin secretion in vivo. *Am J Physiol* 269, F134-9.

Bender, A. T., Silverstein, A. M., Demady, D. R., Kanelakis, K. C., Noguchi, S., Pratt, W. B., and Osawa, Y. (1999). Neuronal nitric-oxide synthase is regulated by the Hsp90-based chaperone system in vivo. *J Biol Chem* 274, 1472-8.

Berlett, B. S., Friguet, B., Yim, M. B., Chock, P. B., and Stadtman, E. R. (1996). Peroxynitrite-mediated nitration of tyrosine residues in *Escherichia coli* glutamine synthetase mimics adenylation: relevance to signal transduction. *Proc Natl Acad Sci U S A* 93, 1776-80.

Bolotina, V. M., Najibi, S., Palacino, J. J., Pagano, P. J., and Cohen, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 368, 850-3.

Bosse, H. M., and Bachmann, S. (1997). Immunohistochemically detected protein nitration indicates sites of renal nitric oxide release in Goldblatt hypertension. *Hypertension* 30, 948-52.

Bosse, H. M., Bohm, R., Resch, S., and Bachmann, S. (1995). Parallel regulation of constitutive NO synthase and renin at JGA of rat kidney under various stimuli. *Am J Physiol* 269, F793-805.

Bredt, D. S., Ferris, C. D., and Snyder, S. H. (1992). Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP- dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase; identification of flavin and calmodulin binding sites. *J Biol Chem* 267, 10976-81.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351, 714-8.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Brecht, D. S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* 84, 757-67.

Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., and Brecht, D. S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82, 743-52.

Brenman, J. E., Xia, H., Chao, D. S., Black, S. M., and Brecht, D. S. (1997). Regulation of neuronal nitric oxide synthase through alternative transcripts. *Dev Neurosci* 19, 224-31.

Brenner, B. M., Meyer, T. W., and Hostetter, T. H. (1982). Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. *N Engl J Med* 307, 652-9.

Breton, S., Lisanti, M. P., Tyszkowski, R., McLaughlin, M., and Brown, D. (1998). Basolateral distribution of caveolin-1 in the kidney. Absence from H<sup>+</sup>-atpase-coated endocytic vesicles in intercalated cells. *J Histochem Cytochem* 46, 205-14.

Burns, K. D., and Harris, R. C. (1995). Signaling and growth responses of LLC-PK1/C14 cells transfected with the rabbit AT1 ANG II receptor. *Am J Physiol* 268, C925-35.

Burns, K. D., Homma, T., and Harris, R. C. (1991). Regulation of Na<sup>(+)</sup>-H<sup>+</sup> exchange by ATP depletion and calmodulin antagonism in renal epithelial cells. *Am J Physiol* 261, F607-16.

Burns, K. D., Inagami, T., and Harris, R. C. (1993). Cloning of a rabbit kidney cortex AT1 angiotensin II receptor that is present in proximal tubule epithelium. *Am J Physiol* 264, F645-54.

Caramelo, C., Lopez-Farre, A., Riesco, A., Olivera, A., Okada, K., Cragoe, E. J., Jr., Tsai, P., Briner, V. A., and Schrier, R. W. (1994). Atrial natriuretic peptide and cGMP inhibit Na<sup>+</sup>/H<sup>+</sup> antiporter in vascular smooth muscle cells in culture. *Kidney Int* 45, 66-75.

Carango, P., Noble, J. E., Marks, H. G., and Funanage, V. L. (1993). Absence of myotonic dystrophy protein kinase (DMPK) mRNA as a result of a triplet repeat expansion in myotonic dystrophy. *Genomics* 18, 340-8.

Chen, P. F., Tsai, A. L., Berka, V., and Wu, K. K. (1996). Endothelial nitric-oxide synthase. Evidence for bidomain structure and successful reconstitution of catalytic activity from two separate domains generated by a baculovirus expression system. *J Biol Chem* 271, 14631-5.

Chen, P. Y., and Sanders, P. W. (1991). L-arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats. *J Clin Invest* 88, 1559-67.

Chen, P. Y., and Sanders, P. W. (1993). Role of nitric oxide synthesis in salt-sensitive hypertension in Dahl/Rapp rats. *Hypertension* 22, 812-8.

Cho, H. J., Xie, Q. W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., and Nathan, C. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med* 176, 599-604.

Christopherson, K. S., and Brecht, D. S. (1997). Nitric oxide in excitable tissues: physiological roles and disease. *J Clin Invest* 100, 2424-9.

Cornwell, T. L., Arnold, E., Boerth, N. J., and Lincoln, T. M. (1994). Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol* 267, C1405-13.

Cowley, A. W., Jr. (1997). Role of the renal medulla in volume and arterial pressure regulation. *Am J Physiol* 273, R1-15.

Cowley, A. W., Jr., and Roman, R. J. (1996). The role of the kidney in hypertension. *Jama* 275, 1581-9.

Cowley, A. W., Roman, R. J., Fenoy, F. J., and Mattson, D. L. (1992). Effect of renal medullary circulation on arterial pressure. *J Hypertens Suppl* 10, S187-93.

Craven, P. A., Studer, R. K., and DeRubertis, F. R. (1994). Impaired nitric oxide-dependent cyclic guanosine monophosphate generation in glomeruli from diabetic rats. Evidence for protein kinase C-mediated suppression of the cholinergic response. *J Clin Invest* 93, 311-20.

Crepieux, P., Kwon, H., Leclerc, N., Spencer, W., Richard, S., Lin, R., and Hiscott, J. (1997). I kappaB alpha physically interacts with a cytoskeleton-associated protein through its signal response domain. *Mol Cell Biol* 17, 7375-85.

Dawson, T. M., Steiner, J. P., Dawson, V. L., Dinerman, J. L., Uhl, G. R., and Snyder, S. H. (1993). Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity [see comments]. *Proc Natl Acad Sci U S A* 90, 9808-12.

De Nicola, L., Blantz, R. C., and Gabbai, F. B. (1992). Nitric oxide and angiotensin II. Glomerular and tubular interaction in the rat. *J Clin Invest* 89, 1248-56.

Degerman, E., Belfrage, P., and Manganiello, V. C. (1997). Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J Biol Chem* 272, 6823-6.

- Delpire, E., Rauchman, M. I., Beier, D. R., Hebert, S. C., and Gullans, S. R. (1994). Molecular cloning and chromosome localization of a putative basolateral Na(+)-K(+)-2Cl<sup>-</sup> cotransporter from mouse inner medullary collecting duct (mIMCD-3) cells. *J Biol Chem* 269, 25677-83.
- Deng, X., Welch, W. J., and Wilcox, C. S. (1994). Renal vasoconstriction during inhibition of NO synthase: effects of dietary salt. *Kidney Int* 46, 639-46.
- Deng, X., Welch, W. J., and Wilcox, C. S. (1995). Renal vasodilation with L-arginine. Effects of dietary salt. *Hypertension* 26, 256-62.
- Deng, X., Welch, W. J., and Wilcox, C. S. (1996). Role of nitric oxide in short-term and prolonged effects of angiotensin II on renal hemodynamics. *Hypertension* 27, 1173-9.
- Denninger, J. W., and Marletta, M. A. (1999). Guanylate cyclase and the .NO/cGMP signaling pathway. *Biochim Biophys Acta* 1411, 334-50.
- Dick, T., Ray, K., Salz, H. K., and Chia, W. (1996). Cytoplasmic dynein (ddlc1) mutations cause morphogenetic defects and apoptotic cell death in *Drosophila melanogaster*. *Mol Cell Biol* 16, 1966-77.
- Dimmeler, S., Lottspeich, F., and Brune, B. (1992). Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 267, 16771-4.
- Dinerman, J. L., Steiner, J. P., Dawson, T. M., Dawson, V., and Snyder, S. H. (1994). Cyclic nucleotide dependent phosphorylation of neuronal nitric oxide synthase inhibits catalytic activity. *Neuropharmacology* 33, 1245-51.
- Drapier, J. C., Hirling, H., Wietzerbin, J., Kaldy, P., and Kuhn, L. C. (1993). Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. *Embo J* 12, 3643-9.

Duc, C., Farman, N., Canessa, C. M., Bonvalet, J. P., and Rossier, B. C. (1994). Cell-specific expression of epithelial sodium channel alpha, beta, and gamma subunits in aldosterone-responsive epithelia from the rat: localization by in situ hybridization and immunocytochemistry. *J Cell Biol* 127, 1907-21.

Eissa, N. T., Strauss, A. J., Haggerty, C. M., Choo, E. K., Chu, S. C., and Moss, J. (1996). Alternative splicing of human inducible nitric-oxide synthase mRNA. tissue-specific regulation and induction by cytokines. *J Biol Chem* 271, 27184-7.

Eitle, E., Harris, P. J., and Morgan, T. O. (1994). Effects of atrial natriuretic factor on cyclic nucleotides in rabbit proximal tubule. *Hypertension* 23, 358-63.

Fan, J. S., Zhang, Q., Li, M., Tochio, H., Yamazaki, T., Shimizu, M., and Zhang, M. (1998). Protein inhibitor of neuronal nitric-oxide synthase, PIN, binds to a 17- amino acid residue fragment of the enzyme. *J Biol Chem* 273, 33472-81.

Fanning, A. S., and Anderson, J. M. (1999). PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. *J Clin Invest* 103, 767-72.

Fenoy, F. J., Ferrer, P., Carbonell, L., and Garcia-Salom, M. (1995). Role of nitric oxide on papillary blood flow and pressure natriuresis. *Hypertension* 25, 408-14.

Feron, O., Saldana, F., Michel, J. B., and Michel, T. (1998). The endothelial nitric-oxide synthase-caveolin regulatory cycle. *J Biol Chem* 273, 3125-8.

Firsov, D., Schild, L., Gautschi, I., Merillat, A. M., Schneeberger, E., and Rossier, B. C. (1996). Cell surface expression of the epithelial Na channel and a mutant causing Liddle syndrome: a quantitative approach. *Proc Natl Acad Sci U S A* 93, 15370-5.

- Floege, J., Burns, M. W., Alpers, C. E., Yoshimura, A., Pritzl, P., Gordon, K., Seifert, R. A., Bowen-Pope, D. F., Couser, W. G., and Johnson, R. J. (1992). Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 41, 297-309.
- Forte, L. R., Thorne, P. K., Eber, S. L., Krause, W. J., Freeman, R. H., Francis, S. H., and Corbin, J. D. (1992). Stimulation of intestinal Cl<sup>-</sup> transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol* 263, C607-15.
- Frey, C., Narayanan, K., McMillan, K., Spack, L., Gross, S. S., Masters, B. S., and Griffith, O. W. (1994). L-thiocitrulline. A stereospecific, heme-binding inhibitor of nitric-oxide synthases. *J Biol Chem* 269, 26083-91.
- Fujihara, C. K., De Nucci, G., and Zatz, R. (1995). Chronic nitric oxide synthase inhibition aggravates glomerular injury in rats with subtotal nephrectomy. *J Am Soc Nephrol* 5, 1498-507.
- Fujisawa, H., Ogura, T., Kurashima, Y., Yokoyama, T., Yamashita, J., and Esumi, H. (1994). Expression of two types of nitric oxide synthase mRNA in human neuroblastoma cell lines. *J Neurochem* 63, 140-5.
- Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373-6.
- Gachhui, R., Abu-Soud, H. M., Ghosha, D. K., Presta, A., Blazing, M. A., Mayer, B., George, S. E., and Stuehr, D. J. (1998). Neuronal nitric-oxide synthase interaction with calmodulin-troponin C chimeras. *J Biol Chem* 273, 5451-4.
- Gachhui, R., Presta, A., Bentley, D. F., Abu-Soud, H. M., McArthur, R., Brudvig, G., Ghosh, D. K., and Stuehr, D. J. (1996). Characterization of the reductase domain of rat neuronal nitric oxide synthase

- generated in the methylotrophic yeast *Pichia pastoris*. Calmodulin response is complete within the reductase domain itself. *J Biol Chem* 271, 20594-602.
- Gamba, G. (1999). Molecular biology of distal nephron sodium transport mechanisms [In Process Citation]. *Kidney Int* 56, 1606-22.
- Gamba, G., Miyanoshita, A., Lombardi, M., Lytton, J., Lee, W. S., Hediger, M. A., and Hebert, S. C. (1994). Molecular cloning, primary structure, and characterization of two members of the mammalian electroneutral sodium-(potassium)-chloride cotransporter family expressed in kidney. *J Biol Chem* 269, 17713-22.
- Gamba, G., Saltzberg, S. N., Lombardi, M., Miyanoshita, A., Lytton, J., Hediger, M. A., Brenner, B. M., and Hebert, S. C. (1993). Primary structure and functional expression of a cDNA encoding the thiazide-sensitive, electroneutral sodium-chloride cotransporter. *Proc Natl Acad Sci U S A* 90, 2749-53.
- Gambaryan, S., Hausler, C., Markert, T., Pohler, D., Jarchau, T., Walter, U., Haase, W., Kurtz, A., and Lohmann, S. M. (1996). Expression of type II cGMP-dependent protein kinase in rat kidney is regulated by dehydration and correlated with renin gene expression. *J Clin Invest* 98, 662-70.
- Gambaryan, S., Wagner, C., Smolenski, A., Walter, U., Poller, W., Haase, W., Kurtz, A., and Lohmann, S. M. (1998). Endogenous or overexpressed cGMP-dependent protein kinases inhibit cAMP-dependent renin release from rat isolated perfused kidney, microdissected glomeruli, and isolated juxtaglomerular cells. *Proc Natl Acad Sci U S A* 95, 9003-8.
- Garcia, N. H., Plato, C. F., Stoos, B. A., and Garvin, J. L. (1999). Nitric oxide-induced inhibition of transport by thick ascending limbs from Dahl salt-sensitive rats. *Hypertension* 34, 508-13.

- Garcia, N. H., Pomposiello, S. I., and Garvin, J. L. (1996). Nitric oxide inhibits ADH-stimulated osmotic water permeability in cortical collecting ducts. *Am J Physiol* 270, F206-10.
- Garcia, N. H., Stoos, B. A., Carretero, O. A., and Garvin, J. L. (1996). Mechanism of the nitric oxide-induced blockade of collecting duct water permeability. *Hypertension* 27, 679-83.
- Garcia-Cardena, G., Martasek, P., Masters, B. S., Skidd, P. M., Couet, J., Li, S., Lisanti, M. P., and Sessa, W. C. (1997). Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J Biol Chem* 272, 25437-40.
- Geibel, J., Giebisch, G., and Boron, W. F. (1990). Angiotensin II stimulates both Na(+)-H+ exchange and Na+/HCO3- cotransport in the rabbit proximal tubule. *Proc Natl Acad Sci U S A* 87, 7917-20.
- Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., and Billiar, T. R. (1993). Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc Natl Acad Sci U S A* 90, 3491-5.
- Geng, Y. J., Almqvist, M., and Hansson, G. K. (1994). cDNA cloning and expression of inducible nitric oxide synthase from rat vascular smooth muscle cells. *Biochim Biophys Acta* 1218, 421-4.
- Gmaj, P., and Murer, H. (1986). Cellular mechanisms of inorganic phosphate transport in kidney. *Physiol Rev* 66, 36-70.
- Goldberg, M. R., Tanaka, W., Barchowsky, A., Bradstreet, T. E., McCrea, J., Lo, M. W., McWilliams, E. J., Jr., and Bjornsson, T. D. (1993). Effects of losartan on blood pressure, plasma renin activity, and angiotensin II in volunteers. *Hypertension* 21, 704-13.

- Green, L. C., Ruiz de Luzuriaga, K., Wagner, D. A., Rand, W., Istfan, N., Young, V. R., and Tannenbaum, S. R. (1981). Nitrate biosynthesis in man. *Proc Natl Acad Sci U S A* 78, 7764-8.
- Green, L. C., Tannenbaum, S. R., and Goldman, P. (1981). Nitrate synthesis in the germfree and conventional rat. *Science* 212, 56-8.
- Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [<sup>15</sup>N]nitrate in biological fluids. *Anal Biochem* 126, 131-8.
- Greenberg, S. G., He, X. R., Schnermann, J. B., and Briggs, J. P. (1995). Effect of nitric oxide on renin secretion. I. Studies in isolated juxtaglomerular granular cells. *Am J Physiol* 268, F948-52.
- Greenwood, M. T., Guo, Y., Kumar, U., Beausejourns, S., and Hussain, S. N. (1997). Distribution of protein inhibitor of neuronal nitric oxide synthase in rat brain. *Biochem Biophys Res Commun* 238, 617-21.
- Grenier, F. C., and Smith, W. L. (1978). Formation of 6-keto-PGF<sub>1</sub> by collecting tubule cells isolated from rabbit renal papillae. *Prostaglandins* 16, 759-772.
- Gross, S. S. (1997). Nitric oxide synthases and their cofactors. In *Nitric oxide and the kidney*, M. S. Goligorsky, and Gross, S. S., ed. (New York: Chapman&Hall), pp. 52-65.
- Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H., and Kedes, L. (1983). Isolation and characterization of full-length cDNA clones for human alpha-, beta-, and gamma-actin mRNAs: skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. *Mol Cell Biol* 3, 787-95.
- Guo, Y., Greenwood, M. T., Petrof, B. J., and Hussain, S. N. (1999). Expression and regulation of protein inhibitor of neuronal nitric oxide synthase in ventilatory muscles. *Am J Respir Cell Mol Biol* 20, 319-26.

Guyton, A., C. (1991). Textbook of medical physiology, 8th Edition, M. Wonsiewicz, J, ed. (Philadelphia: W. B. Saunders).

Guyton, A. C. (1991). Blood pressure control--special role of the kidneys and body fluids. *Science* 252, 1813-6.

Guzman, N. J., Fang, M. Z., Tang, S. S., Ingelfinger, J. R., and Garg, L. C. (1995). Autocrine inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase by nitric oxide in mouse proximal tubule epithelial cells. *J Clin Invest* 95, 2083-8.

Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C. L., and Marsden, P. A. (1994). Structural organization of the human neuronal nitric oxide synthase gene (NOS1). *J Biol Chem* 269, 33082-90.

Hall, J. E., Brands, M. W., and Henegar, J. R. (1999). Angiotensin II and long-term arterial pressure regulation: the overriding dominance of the kidney. *J Am Soc Nephrol* 10 Suppl 12, S258-65.

Hamm-Alvarez, S. F., and Sheetz, M. P. (1998). Microtubule-dependent vesicle transport: modulation of channel and transporter activity in liver and kidney. *Physiol Rev* 78, 1109-29.

Hammond, T. G., Yusufi, A. N., Knox, F. G., and Dousa, T. P. (1985). Administration of atrial natriuretic factor inhibits sodium-coupled transport in proximal tubules. *J Clin Invest* 75, 1983-9.

Harrison, S. C. (1996). Peptide-surface association: the case of PDZ and PTB domains. *Cell* 86, 341-3.

Haynes, W. G., Hand, M. F., Dockrell, M. E., Eadington, D. W., Lee, M. R., Hussein, Z., Benjamin, N., and Webb, D. J. (1997). Physiological role of nitric oxide in regulation of renal function in humans. *Am J Physiol* 272, F364-71.

- He, X. R., Greenberg, S. G., Briggs, J. P., and Schnermann, J. B. (1995). Effect of nitric oxide on renin secretion. II. Studies in the perfused juxtaglomerular apparatus. *Am J Physiol* 268, F953-9.
- Hecker, M., Sessa, W. C., Harris, H. J., Anggard, E. E., and Vane, J. R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc Natl Acad Sci U S A* 87, 8612-6.
- Hein, L., Barsh, G. S., Pratt, R. E., Dzau, V. J., and Kobilka, B. K. (1995). Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice [published erratum appears in *Nature* 1996 Mar 28;380(6572):366]. *Nature* 377, 744-7.
- Hemmens, B., Woschitz, S., Pitters, E., Klosch, B., Volker, C., Schmidt, K., and Mayer, B. (1998). The protein inhibitor of neuronal nitric oxide synthase (PIN): characterization of its action on pure nitric oxide synthases. *FEBS Lett* 430, 397-400.
- Hennington, B. S., Zhang, H., Miller, M. T., Granger, J. P., and Reckelhoff, J. F. (1998). Angiotensin II stimulates synthesis of endothelial nitric oxide synthase. *Hypertension* 31, 283-8.
- Higashi, Y., Oshima, T., Watanabe, M., Matsuura, H., and Kajiyama, G. (1996). Renal response to L-arginine in salt-sensitive patients with essential hypertension. *Hypertension* 27, 643-8.
- Hirokawa, N., Noda, Y., and Okada, Y. (1998). Kinesin and dynein superfamily proteins in organelle transport and cell division. *Curr Opin Cell Biol* 10, 60-73.
- Hostetter, T. H., Olson, J. L., Rennke, H. G., Venkatachalam, M. A., and Brenner, B. M. (1981). Hyperfiltration in remnant nephrons: a potentially adverse response to renal ablation. *Am J Physiol* 241, F85-93.

- Hu, L., and Manning, R. D., Jr. (1995). Role of nitric oxide in regulation of long-term pressure-natriuresis relationship in Dahl rats. *Am J Physiol* 268, H2375-83.
- Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H., and Fishman, M. C. (1993). Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75, 1273-86.
- Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A., and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase [see comments]. *Nature* 377, 239-42.
- Huang, Z., Huang, P. L., Panahian, N., Dalkara, T., Fishman, M. C., and Moskowitz, M. A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265, 1883-5.
- Husted, R. F., Takahashi, T., and Stokes, J. B. (1996). IMCD cells cultured from Dahl S rats absorb more Na<sup>+</sup> than Dahl R rats. *Am J Physiol* 271, F1029-36.
- Ichihara, A., Imig, J. D., Inscho, E. W., and Navar, L. G. (1998). Interactive nitric oxide-angiotensin II influences on renal microcirculation in angiotensin II-induced hypertension. *Hypertension* 31, 1255-60.
- Ichihara, A., Inscho, E. W., Imig, J. D., and Navar, L. G. (1998). Neuronal nitric oxide synthase modulates rat renal microvascular function. *Am J Physiol* 274, F516-24.
- Ichiki, T., Labosky, P. A., Shiota, C., Okuyama, S., Imagawa, Y., Fogo, A., Niimura, F., Ichikawa, I., Hogan, B. L., and Inagami, T. (1995). Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature* 377, 748-50.

Idriss, S. D., Gudi, T., Casteel, D. E., Kharitonov, V. G., Pilz, R. B., and Boss, G. R. (1999). Nitric oxide regulation of gene transcription via soluble guanylate cyclase and type I cGMP-dependent protein kinase. *J Biol Chem* 274, 9489-93.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 84, 9265-9.

Ikeda, Y., Saito, K., Kim, J. I., and Yokoyama, M. (1995). Nitric oxide synthase isoform activities in kidney of Dahl salt- sensitive rats. *Hypertension* 26, 1030-4.

Ishii, K., Chang, B., Kerwin, J. F., Jr., Wagenaar, F. L., Huang, Z. J., and Murad, F. (1991). Formation of endothelium-derived relaxing factor in porcine kidney epithelial LLC-PK1 cells: an intra- and intercellular messenger for activation of soluble guanylate cyclase. *J Pharmacol Exp Ther* 256, 38-43.

Ito, S., Arima, S., Ren, Y. L., Juncos, L. A., and Carretero, O. A. (1993). Endothelium-derived relaxing factor/nitric oxide modulates angiotensin II action in the isolated microperfused rabbit afferent but not efferent arteriole. *J Clin Invest* 91, 2012-9.

Ito, S., Johnson, C. S., and Carretero, O. A. (1991). Modulation of angiotensin II-induced vasoconstriction by endothelium- derived relaxing factor in the isolated microperfused rabbit afferent arteriole. *J Clin Invest* 87, 1656-63.

Ito, S., and Ren, Y. (1993). Evidence for the role of nitric oxide in macula densa control of glomerular hemodynamics. *J Clin Invest* 92, 1093-8.

Jaffrey, S. R., and Snyder, S. H. (1996). PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science* 274, 774-7.

Janssens, S. P., Simouchi, A., Quertermous, T., Bloch, D. B., and Bloch, K. D. (1992). Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. *J Biol Chem* 267, 22694.

Jeong, Y., Won, J., and Yim, J. (1998). Cloning and structure of a rabbit protein inhibitor of neuronal nitric oxide synthase (PIN) gene and its pseudogene. *Gene* 214, 67-75.

Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996). S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control [see comments]. *Nature* 380, 221-6.

Johnson, R. J., Alpers, C. E., Yoshimura, A., Lombardi, D., Pritzl, P., Floege, J., and Schwartz, S. M. (1992). Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19, 464-74.

Junaid, A., Rosenberg, M. E., and Hostetter, T. H. (1997). Interaction of angiotensin II and TGF-beta I in the rat remnant kidney [published erratum appears in *J Am Soc Nephrol* 1998 Jan;9(1):154]. *J Am Soc Nephrol* 8, 1732-8.

Kagami, S., Border, W. A., Miller, D. E., and Noble, N. A. (1994). Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest* 93, 2431-7.

Kelm, M. (1999). Nitric oxide metabolism and breakdown. *Biochim Biophys Acta* 1411, 273-89.

Khatsenko, O. G., Gross, S. S., Rifkind, A. B., and Vane, J. R. (1993). Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. *Proc Natl Acad Sci U S A* 90, 11147-51.

Khraibi, A. A. (1994). Inhibition of nitric oxide causes exaggerated natriuresis in spontaneously hypertensive rats. *Am J Physiol* 266, F762-6.

King, S. M., Barbarese, E., Dillman, J. F., III, Patel-King, R. S., Carson, J. H., and Pfister, K. K. (1996). Brain cytoplasmic and flagellar outer arm dyneins share a highly conserved Mr 8,000 light chain. *J Biol Chem* 271, 19358-66.

King, S. M., and Patel-King, R. S. (1995). The M(r) = 8,000 and 11,000 outer arm dynein light chains from *Chlamydomonas flagella* have cytoplasmic homologues. *J Biol Chem* 270, 11445-52.

Kirchner, K. A., Crosby, B. A., Patel, A. R., and Granger, J. P. (1995). Segmental chloride transport in the Dahl-S rat kidney during L-arginine administration. *J Am Soc Nephrol* 5, 1567-72.

Klatt, P., Molina, E. P., and Lamas, S. (1999). Nitric oxide inhibits c-Jun DNA binding by specifically targeted S- glutathionylation. *J Biol Chem* 274, 15857-64.

Knox, F. G., Burnett, J. C., Jr., Kohan, D. E., Spielman, W. S., and Strand, J. C. (1980). Escape from the sodium-retaining effects of mineralocorticoids. *Kidney Int* 17, 263-76.

Kohan, D. E., Hughes, A. K., and Perkins, S. L. (1992). Characterization of endothelin receptors in the inner medullary collecting duct of the rat. *J Biol Chem* 267, 12336-40.

Kone, B. C. (1999). Localization and regulation of nitric oxide synthase isoforms in the kidney [In Process Citation]. *Semin Nephrol* 19, 230-41.

Kone, B. C. (1997). Nitric oxide in renal health and disease. *Am J Kidney Dis* 30, 311-33.

- Kone, B. C., and Baylis, C. (1997). Biosynthesis and homeostatic roles of nitric oxide in the normal kidney. *Am J Physiol* 272 (*Renal Physiol.* 41), F561-78.
- Kone, B. C., and Higham, S. (1999). Nitric oxide inhibits transcription of the Na<sup>+</sup>-K<sup>+</sup>-ATPase alpha1-subunit gene in an MTAL cell line. *Am J Physiol* 276, F614-21.
- Kontogiannis, J., and Burns, K. D. (1998). Role of AT1 angiotensin II receptors in renal ischemic injury. *Am J Physiol* 274, F79-90.
- Kroncke, K. D., Fehsel, K., and Kolb-Bachofen, V. (1995). Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. *Biol Chem Hoppe Seyler* 376, 327-43.
- Kurashima, K., Yu, F. H., Cabado, A. G., Szabo, E. Z., Grinstein, S., and Orłowski, J. (1997). Identification of sites required for down-regulation of Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 activity by cAMP-dependent protein kinase. phosphorylation-dependent and -independent mechanisms. *J Biol Chem* 272, 28672-9.
- Kurtz, A., Gotz, K. H., Hamann, M., and Wagner, C. (1998). Stimulation of renin secretion by nitric oxide is mediated by phosphodiesterase 3. *Proc Natl Acad Sci U S A* 95, 4743-7.
- Kurtz, A., and Wagner, C. (1998). Role of nitric oxide in the control of renin secretion. *Am J Physiol* 275, F849-62.
- Lafont, F., Burkhardt, J. K., and Simons, K. (1994). Involvement of microtubule motors in basolateral and apical transport in kidney cells. *Nature* 372, 801-3.
- Lahera, V., Navarro, J., Biondi, M. L., Ruilope, L. M., and Romero, J. C. (1993). Exogenous cGMP prevents decrease in diuresis and natriuresis induced by inhibition of NO synthesis. *Am J Physiol* 264, F344-7.

- Lahera, V., Salom, M. G., Miranda-Guardiola, F., Moncada, S., and Romero, J. C. (1991). Effects of NG-nitro-L-arginine methyl ester on renal function and blood pressure. *Am J Physiol* 261, F1033-7.
- Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992). Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc Natl Acad Sci U S A* 89, 6348-52.
- Lamprecht, G., Weinman, E. J., and Yun, C. H. (1998). The role of NHERF and E3KARP in the cAMP-mediated inhibition of NHE3. *J Biol Chem* 273, 29972-8.
- Lander, H. M., Hajjar, D. P., Hempstead, B. L., Mirza, U. A., Chait, B. T., Campbell, S., and Quilliam, L. A. (1997). A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. *J Biol Chem* 272, 4323-6.
- Lander, H. M., Jacovina, A. T., Davis, R. J., and Tauras, J. M. (1996). Differential activation of mitogen-activated protein kinases by nitric oxide-related species. *J Biol Chem* 271, 19705-9.
- Lander, H. M., Ogiste, J. S., Pearce, S. F., Levi, R., and Novogrodsky, A. (1995). Nitric oxide-stimulated guanine nucleotide exchange on p21ras. *J Biol Chem* 270, 7017-20.
- Lane, P., and Gross, S. S. (1999). Cell signaling by nitric oxide. *Semin Nephrol* 19, 215-29.
- Lee, L. K., Meyer, T. W., Pollock, A. S., and Lovett, D. H. (1995). Endothelial cell injury initiates glomerular sclerosis in the rat remnant kidney. *J Clin Invest* 96, 953-64.

- Levine, D. Z., Iacovitti, M., Buckman, S., Hincke, M. T., Luck, B., and Fryer, J. N. (1997). ANG II-dependent HCO<sub>3</sub><sup>-</sup> reabsorption in surviving rat distal tubules: expression/activation of H(+)-ATPase. *Am J Physiol* 272, F799-808.
- Liang, M., and Knox, F. G. (1999). Nitric oxide reduces the molecular activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase in opossum kidney cells. *Kidney Int* 56, 627-34.
- Lifton, R. P. (1996). Molecular genetics of human blood pressure variation. *Science* 272, 676-80.
- Linaz, S. L., and Repine, J. E. (1999). Endothelial cells regulate proximal tubule epithelial cell sodium transport. *Kidney Int* 55, 1251-8.
- Lippoldt, A., Gross, V., Schneider, K., Hansson, A., Nadaud, S., Schneider, W., Bader, M., Yagil, C., Yagil, Y., and Luft, F. C. (1997). Nitric oxide synthase and renin-angiotensin system gene expression in salt-sensitive and salt-resistant Sabra rats. *Hypertension* 30, 409-15.
- Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993). A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds [see comments]. *Nature* 364, 626-32.
- Lo, M., Liu, K. L., Lantelme, P., and Sassard, J. (1995). Subtype 2 of angiotensin II receptors controls pressure-natriuresis in rats. *J Clin Invest* 95, 1394-7.
- Lohmann, S. M., Vaandrager, A. B., Smolenski, A., Walter, U., and De Jonge, H. R. (1997). Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem Sci* 22, 307-12.
- Lortie, M. J., Mendonca, M., and Blantz, R. C. (1994). Hormone mediated cGMP generation in isolated proximal tubules. *J Am Soc Nephrol* 5, 663.

Lowenstein, C. J., Glatt, C. S., Bredt, D. S., and Snyder, S. H. (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc Natl Acad Sci U S A* 89, 6711-5.

Lyons, C. R., Orloff, G. J., and Cunningham, J. M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J Biol Chem* 267, 6370-4.

MacMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q. W., Sokol, K., Hutchinson, N., and et al. (1995). Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase [published erratum appears in *Cell* 1995 Jun 30;81(7):following 1170]. *Cell* 81, 641-50.

Madrid, M. I., Garcia-Salom, M., Tornel, J., de Gasparo, M., and Fenoy, F. J. (1997). Interactions between nitric oxide and angiotensin II on renal cortical and papillary blood flow. *Hypertension* 30, 1175-82.

Majid, D. S., Williams, A., and Navar, L. G. (1993). Inhibition of nitric oxide synthesis attenuates pressure-induced natriuretic responses in anesthetized dogs. *Am J Physiol* 264, F79-87.

Malinski, T., and Taha, Z. (1992). Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor [see comments]. *Nature* 358, 676-8.

Markewitz, B. A., Michael, J. R., and Kohan, D. E. (1993). Cytokine-induced expression of a nitric oxide synthase in rat renal tubule cells. *J Clin Invest* 91, 2138-43.

Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D., and Wishnok, J. S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* 27, 8706-11.

Marples, D., Schroer, T. A., Ahrens, N., Taylor, A., Knepper, M. A., and Nielsen, S. (1998). Dynein and dynactin colocalize with AQP2 water channels in intracellular vesicles from kidney collecting duct. *Am J Physiol* 274 (*Renal Physiol.* 43), F384-94.

Marsden, P. A., Schappert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S., and Michel, T. (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett* 307, 287-93.

Martin, B. L., Wu, D., Jakes, S., and Graves, D. J. (1990). Chemical influences on the specificity of tyrosine phosphorylation. *J Biol Chem* 265, 7108-11.

Masters, B. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., and Martasek, P. (1996). Neuronal nitric oxide synthase, a modular enzyme formed by convergent evolution: structure studies of a cysteine thiolate-liganded heme protein that hydroxylates L-arginine to produce NO. as a cellular signal [published erratum appears in *FASEB J* 1996 Jul;10(9):1107]. *Faseb J* 10, 552-8.

Matsuoka, H., Itoh, S., Kimoto, M., Kohno, K., Tamai, O., Wada, Y., Yasukawa, H., Iwami, G., Okuda, S., and Imaizumi, T. (1997). Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension. *Hypertension* 29, 242-7.

Mattson, D. L., and Bellehumeur, T. G. (1996). Neural nitric oxide synthase in the renal medulla and blood pressure regulation. *Hypertension* 28, 297-303.

Mattson, D. L., and Higgins, D. J. (1996). Influence of dietary sodium intake on renal medullary nitric oxide synthase. *Hypertension* 27, 688-92.

Mattson, D. L., Lu, S., and Cowley, A. W., Jr. (1997). Role of nitric oxide in the control of the renal medullary circulation. *Clin Exp Pharmacol Physiol* 24, 587-90.

Mattson, D. L., Lu, S., Nakanishi, K., Papanek, P. E., and Cowley, A. W., Jr. (1994). Effect of chronic renal medullary nitric oxide inhibition on blood pressure. *Am J Physiol* 266, H1918-26.

Mattson, D. L., Maeda, C. Y., Bachman, T. D., and Cowley, A. W., Jr. (1998). Inducible nitric oxide synthase and blood pressure. *Hypertension* 31, 15-20.

Mattson, D. L., Roman, R. J., and Cowley, A. W., Jr. (1992). Role of nitric oxide in renal papillary blood flow and sodium excretion. *Hypertension* 19, 766-9.

McKee, M., Scavone, C., and Nathanson, J. A. (1994). Nitric oxide, cGMP, and hormone regulation of active sodium transport. *Proc Natl Acad Sci U S A* 91, 12056-60.

McLay, J. S., Chatterjee, P., Nicolson, A. G., Jardine, A. G., McKay, N. G., Ralston, S. H., Grabowski, P., Haites, N. E., MacLeod, A. M., and Hawksworth, G. M. (1994). Nitric oxide production by human proximal tubular cells: a novel immunomodulatory mechanism? *Kidney Int* 46, 1043-9.

Michel, R. N., Vu, C. Q., Tetzlaff, W., and Jasmin, B. J. (1994). Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses. *J Cell Biol* 127, 1061-9.

Michel, T., and Feron, O. (1997). Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 100, 2146-52.

Miyata, N., and Cowley, A. W., Jr. (1999). Renal intramedullary infusion of L-arginine prevents reduction of medullary blood flow and hypertension in Dahl salt-sensitive rats. *Hypertension* 33, 446-50.

Miyata, N., Zou, A. P., Mattson, D. L., and Cowley, A. W., Jr. (1998). Renal medullary interstitial infusion of L-arginine prevents hypertension in Dahl salt-sensitive rats. *Am J Physiol* 275, R1667-73.

- Mohaupt, M. G., Elzie, J. L., Ahn, K. Y., Clapp, W. L., Wilcox, C. S., and Kone, B. C. (1994). Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. *Kidney Int* 46, 653-65.
- Mohaupt, M. G., Schwobel, J., Elzie, J. L., Kannan, G. S., and Kone, B. C. (1995). Cytokines activate inducible nitric oxide synthase gene transcription in inner medullary collecting duct cells. *Am J Physiol* 268, F770-7.
- Mount, D. B., Baekgaard, A., Hall, A. E., Plata, C., Xu, J., Beier, D. R., Gamba, G., and Hebert, S. C. (1999). Isoforms of the Na-K-2Cl cotransporter in murine TAL I. Molecular characterization and intrarenal localization. *Am J Physiol* 276, F347-58.
- Nadler, S. P., Zimpelmann, J. A., and Hebert, R. L. (1992). PGE2 inhibits water permeability at a post-cAMP site in rat terminal inner medullary collecting duct. *Am J Physiol* 262, F229-35.
- Nagano, F., Orita, S., Sasaki, T., Naito, A., Sakaguchi, G., Maeda, M., Watanabe, T., Kominami, E., Uchiyama, Y., and Takai, Y. (1998). Interaction of Doc2 with tctex-1, a light chain of cytoplasmic dynein. Implication in dynein-dependent vesicle transport. *J Biol Chem* 273, 30065-8.
- Nakamura, T., Alberola, A. M., and Granger, J. P. (1993). Role of renal interstitial pressure as a mediator of sodium retention during systemic blockade of nitric oxide. *Hypertension* 21, 956-60.
- Nakayama, I., Kawahara, Y., Tsuda, T., Okuda, M., and Yokoyama, M. (1994). Angiotensin II inhibits cytokine-stimulated inducible nitric oxide synthase expression in vascular smooth muscle cells. *J Biol Chem* 269, 11628-33.

- Navar, L. G., Inscho, E. W., Majid, S. A., Imig, J. D., Harrison-Bernard, L. M., and Mitchell, K. D. (1996). Paracrine regulation of the renal microcirculation. *Physiol Rev* 76, 425-536.
- Nelson, R. J., Demas, G. E., Huang, P. L., Fishman, M. C., Dawson, V. L., Dawson, T. M., and Snyder, S. H. (1995). Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase [see comments]. *Nature* 378, 383-6.
- Nishida, C. R., and Ortiz de Montellano, P. R. (1999). Autoinhibition of endothelial nitric-oxide synthase. Identification of an electron transfer control element. *J Biol Chem* 274, 14692-8.
- Nunoshiba, T., deRojas-Walker, T., Wishnok, J. S., Tannenbaum, S. R., and Demple, B. (1993). Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. *Proc Natl Acad Sci U S A* 90, 9993-7.
- Oberbaumer, I., Moser, D., and Bachmann, S. (1998). Nitric oxide synthase 1 mRNA: tissue-specific variants from rat with alternative first exons. *Biol Chem* 379, 913-9.
- Ogura, T., Yokoyama, T., Fujisawa, H., Kurashima, Y., and Esumi, H. (1993). Structural diversity of neuronal nitric oxide synthase mRNA in the nervous system. *Biochem Biophys Res Commun* 193, 1014-22.
- Ollerstam, A., Pittner, J., Persson, A. E., and Thorup, C. (1997). Increased blood pressure in rats after long-term inhibition of the neuronal isoform of nitric oxide synthase. *J Clin Invest* 99, 2212-8.
- Orsio, S., Benigni, A., Bruzzi, I., Corna, D., Perico, N., Zoja, C., Benatti, L., and Remuzzi, G. (1993). Renal endothelin gene expression is increased in remnant kidney and correlates with disease progression. *Kidney Int* 43, 354-8.

- Ozono, R., Wang, Z. Q., Moore, A. F., Inagami, T., Siragy, H. M., and Carey, R. M. (1997). Expression of the subtype 2 angiotensin (AT2) receptor protein in rat kidney. *Hypertension* 30, 1238-46.
- Palmer, R. M., Ashton, D. S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333, 664-6.
- Palmer, R. M., Ferrige, A. G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327, 524-6.
- Pantopoulos, K., and Hentze, M. W. (1995). Nitric oxide signaling to iron-regulatory protein: direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. *Proc Natl Acad Sci U S A* 92, 1267-71.
- Payne, J. A., and Forbush, B., 3rd (1994). Alternatively spliced isoforms of the putative renal Na-K-Cl cotransporter are differentially distributed within the rabbit kidney. *Proc Natl Acad Sci U S A* 91, 4544-8.
- Radermacher, J., Klanke, B., Schurek, H. J., Stolte, H. F., and Frolich, J. C. (1992). Importance of NO/EDRF for glomerular and tubular function: studies in the isolated perfused rat kidney. *Kidney Int* 41, 1549-59.
- Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 266, 4244-50.
- Ranjan, V., Xiao, Z., and Diamond, S. L. (1995). Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. *Am J Physiol* 269, H550-5.
- Roczniak, A., Fryer, J. N., Levine, D. Z., and Burns, K. D. (1999). Downregulation of neuronal nitric oxide synthase in the rat remnant kidney. *J Am Soc Nephrol* 10, 704-13.

- Roczniak, A., Zimpelmann, J., and Burns, K. D. (1998). Effect of dietary salt on neuronal nitric oxide synthase in the inner medullary collecting duct. *Am J Physiol* 275 (*Renal Physiol.* 44), F46-54.
- Rosenberg, M. E., Correa-Rotter, R., Inagami, T., Kren, S. M., and Hostetter, T. H. (1991). Glomerular renin synthesis and storage in the remnant kidney in the rat. *Kidney Int* 40, 677-83.
- Rosenberg, M. E., Smith, L. J., Correa-Rotter, R., and Hostetter, T. H. (1994). The paradox of the renin-angiotensin system in chronic renal disease. *Kidney Int* 45, 403-10.
- Rossier, B. C. (1997). 1996 Homer Smith Award Lecture. Cum grano salis: the epithelial sodium channel and the control of blood pressure. *J Am Soc Nephrol* 8, 980-92.
- Rostand, S. G., Brunzell, J. D., Cannon, R. O. d., and Victor, R. G. (1991). Cardiovascular complications in renal failure [editorial]. *J Am Soc Nephrol* 2, 1053-62.
- Ruan, J., Xie, Q., Hutchinson, N., Cho, H., Wolfe, G. C., and Nathan, C. (1996). Inducible nitric oxide synthase requires both the canonical calmodulin-binding domain and additional sequences in order to bind calmodulin and produce nitric oxide in the absence of free Ca<sup>2+</sup>. *J Biol Chem* 271, 22679-86.
- Rutenburg, A. M. (1966). Histochemical demonstration of alkaline phosphatase using naphthol AS phosphate: comparison with the substituted naphthol AS phosphates. *Ann Histochem* 11, 139-49.
- Salazar, F. J., Alberola, A., Pinilla, J. M., Romero, J. C., and Quesada, T. (1993). Salt-induced increase in arterial pressure during nitric oxide synthesis inhibition. *Hypertension* 22, 49-55.

- Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M., Martasek, P., Roman, L. J., Masters, B. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997). An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. *J Biol Chem* 272, 29769-77.
- Salom, M. G., Lahera, V., Miranda-Guardiola, F., and Romero, J. C. (1992). Blockade of pressure natriuresis induced by inhibition of renal synthesis of nitric oxide in dogs. *Am J Physiol* 262, F718-22.
- Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A* 90, 7240-4.
- Sanders, P. W. (1996). Salt-sensitive hypertension: lessons from animal models. *Am J Kidney Dis* 28, 775-82.
- Schild, L., Canessa, C. M., Shimkets, R. A., Gautschi, I., Lifton, R. P., and Rossier, B. C. (1995). A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the *Xenopus laevis* oocyte expression system. *Proc Natl Acad Sci U S A* 92, 5699-703.
- Schmidt, H. H., Lohmann, S. M., and Walter, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim Biophys Acta* 1178, 153-75.
- Schmidt, R. J., Yokota, S., Tracy, T. S., Sorkin, M. I., and Baylis, C. (1999). Nitric oxide production is low in end-stage renal disease patients on peritoneal dialysis. *Am J Physiol* 276, F794-7.
- Schnermann, J. (1998). Juxtaglomerular cell complex in the regulation of renal salt excretion. *Am J Physiol* 274, R263-79.
- Schnermann, J. B., and Sayegh, S. I. (1998). *Kidney Physiology*, G. W. F, ed. (Philadelphia: Lippincott-Raven).

Scholz, H., and Kurtz, A. (1993). Involvement of endothelium-derived relaxing factor in the pressure control of renin secretion from isolated perfused kidney. *J Clin Invest* 91, 1088-94.

Schricker, K., Hegyi, I., Hamann, M., Kaissling, B., and Kurtz, A. (1995). Tonic stimulation of renin gene expression by nitric oxide is counteracted by tonic inhibition through angiotensin II. *Proc Natl Acad Sci U S A* 92, 8006-10.

Schricker, K., and Kurtz, A. (1993). Liberators of NO exert a dual effect on renin secretion from isolated mouse renal juxtaglomerular cells. *Am J Physiol* 265, F180-6.

Schricker, K., Potzl, B., Hamann, M., and Kurtz, A. (1996). Coordinate changes of renin and brain-type nitric-oxide-synthase (b- NOS) mRNA levels in rat kidneys. *Pflugers Arch* 432, 394-400.

Schultheis, P. J., Clarke, L. L., Meneton, P., Miller, M. L., Soleimani, M., Gawenis, L. R., Riddle, T. M., Duffy, J. J., Doetschman, T., Wang, T., Giebisch, G., Aronson, P. S., Lorenz, J. N., and Shull, G. E. (1998). Renal and intestinal absorptive defects in mice lacking the NHE3 Na<sup>+</sup>/H<sup>+</sup> exchanger. *Nat Genet* 19, 282-5.

Semrad, C. E., Cragoe, E. J., Jr., and Chang, E. B. (1990). Inhibition of Na/H exchange in avian intestine by atrial natriuretic factor. *J Clin Invest* 86, 585-91.

Sessa, W. C., Harrison, J. K., Barber, C. M., Zeng, D., Durieux, M. E., D'Angelo, D. D., Lynch, K. R., and Peach, M. J. (1992). Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J Biol Chem* 267, 15274-6.

Sessa, W. C., Hecker, M., Mitchell, J. A., and Vane, J. R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. *Proc Natl Acad Sci U S A* 87, 8607-11.

Shibuki, K., and Okada, D. (1991). Endogenous nitric oxide release required for long-term synaptic depression in the cerebellum. *Nature* 349, 326-8.

Shultz, P. J., and Tolins, J. P. (1993). Adaptation to increased dietary salt intake in the rat. Role of endogenous nitric oxide. *J Clin Invest* 91, 642-50.

Siddhanta, U., Presta, A., Fan, B., Wolan, D., Rousseau, D. L., and Stuehr, D. J. (1998). Domain swapping in inducible nitric-oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer. *J Biol Chem* 273, 18950-8.

Sigmon, D. H., Newman, J. M., and Beierwaltes, W. H. (1994). Angiotensin II: endothelium-derived nitric oxide interaction in conscious rats. *J Am Soc Nephrol* 4, 1675-82.

Silvagno, F., Xia, H., and Brecht, D. S. (1996). Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J Biol Chem* 271, 11204-8.

Simchon, S., Manger, W., Blumberg, G., Brensilver, J., and Cortell, S. (1996). Impaired renal vasodilation and urinary cGMP excretion in Dahl salt-sensitive rats. *Hypertension* 27, 653-7.

Simon, D. B., Karet, F. E., Hamdan, J. M., DiPietro, A., Sanjad, S. A., and Lifton, R. P. (1996). Bartter's syndrome, hypokalaemic alkalosis with hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter NKCC2. *Nat Genet* 13, 183-8.

Simon, D. B., Nelson-Williams, C., Bia, M. J., Ellison, D., Karet, F. E., Molina, A. M., Vaara, I., Iwata, F., Cushner, H. M., Koolen, M., Gainza, F. J., Gitleman, H. J., and Lifton, R. P. (1996). Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* 12, 24-30.

Singh, I., Grams, M., Wang, W. H., Yang, T., Killen, P., Smart, A., Schnermann, J., and Briggs, J. P. (1996). Coordinate regulation of renal expression of nitric oxide synthase, renin, and angiotensinogen mRNA by dietary salt. *Am J Physiol* 270, F1027-37.

Siragy, H. M., and Carey, R. M. (1997). The subtype 2 (AT<sub>2</sub>) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest* 100, 264-9.

Siragy, H. M., Inagami, T., Ichiki, T., and Carey, R. M. (1999). Sustained hypersensitivity to angiotensin II and its mechanism in mice lacking the subtype-2 (AT<sub>2</sub>) angiotensin receptor. *Proc Natl Acad Sci U S A* 96, 6506-10.

Snyder, P. M., Price, M. P., McDonald, F. J., Adams, C. M., Volk, K. A., Zeiher, B. G., Stokes, J. B., and Welsh, M. J. (1995). Mechanism by which Liddle's syndrome mutations increase activity of a human epithelial Na<sup>+</sup> channel. *Cell* 83, 969-78.

Southam, E., and Garthwaite, J. (1991). Comparative effects of some nitric oxide donors on cyclic GMP levels in rat cerebellar slices. *Neurosci Lett* 130, 107-11.

Stadler, J., Trockfeld, J., Schmalix, W. A., Brill, T., Siewert, J. R., Greim, H., and Doehmer, J. (1994). Inhibition of cytochromes P4501A by nitric oxide. *Proc Natl Acad Sci U S A* 91, 3559-63.

Stamler, J. S. (1994). Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* 78, 931-6.

Stamler, J. S., Jaraki, O., Osborne, J., Simon, D. I., Keaney, J., Vita, J., Singel, D., Valeri, C. R., and Loscalzo, J. (1992). Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci U S A* 89, 7674-7.

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992). S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci U S A* 89, 444-8.

Stoos, B. A., Carretero, O. A., Farhy, R. D., Scicli, G., and Garvin, J. L. (1992). Endothelium-derived relaxing factor inhibits transport and increases cGMP content in cultured mouse cortical collecting duct cells. *J Clin Invest* 89, 761-5.

Stoos, B. A., Carretero, O. A., and Garvin, J. L. (1994). Endothelial-derived nitric oxide inhibits sodium transport by affecting apical membrane channels in cultured collecting duct cells. *J Am Soc Nephrol* 4, 1855-60.

Stoos, B. A., Garcia, N. H., and Garvin, J. L. (1995). Nitric oxide inhibits sodium reabsorption in the isolated perfused cortical collecting duct. *J Am Soc Nephrol* 6, 89-94.

Stuehr, D. J. (1999). Mammalian nitric oxide synthases. *Biochim Biophys Acta* 1411, 217-30.

Stuehr, D. J., and Ikeda-Saito, M. (1992). Spectral characterization of brain and macrophage nitric oxide synthases. Cytochrome P-450-like heme proteins that contain a flavin semiquinone radical. *J Biol Chem* 267, 20547-50.

Subczynski, W. K., Lomnicka, M., and Hyde, J. S. (1996). Permeability of nitric oxide through lipid bilayer membranes. *Free Radic Res* 24, 343-9.

Taddei, S., Virdis, A., Ghiadoni, L., and Salvetti, A. (1998). The role of endothelium in human hypertension. *Curr Opin Nephrol Hypertens* 7, 203-9.

- Tan, D. Y., Meng, S., and Manning, R. D., Jr. (1999). Role of neuronal nitric oxide synthase in Dahl salt-sensitive hypertension. *Hypertension* 33, 456-61.
- Terada, Y., Tomita, K., Nonoguchi, H., and Marumo, F. (1992). Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylate cyclase messenger RNAs in microdissected rat nephron segments. *J Clin Invest* 90, 659-65.
- Terzi, F., Burtin, M., and Friedlander, G. (1998). Early molecular mechanisms in the progression of renal failure: role of growth factors and protooncogenes. *Kidney Int Suppl* 65, S68-73.
- Thorup, C., Kornfeld, M., Goligorsky, M. S., and Moore, L. C. (1999). AT1 receptor inhibition blunts angiotensin II-stimulated nitric oxide release in renal arteries. *J Am Soc Nephrol* 10 Suppl 11, S220-4.
- Thorup, C., Kornfeld, M., Winaver, J. M., Goligorsky, M. S., and Moore, L. C. (1998). Angiotensin-II stimulates nitric oxide release in isolated perfused renal resistance arteries. *Pflugers Arch* 435, 432-4.
- Tojo, A., Bredt, D. S., and Wilcox, C. S. (1999). Distribution of postsynaptic density proteins in rat kidney: relationship to neuronal nitric oxide synthase [In Process Citation]. *Kidney Int* 55, 1384-94.
- Tojo, A., Gross, S. S., Zhang, L., Tisher, C. C., Schmidt, H. H., Wilcox, C. S., and Madsen, K. M. (1994). Immunocytochemical localization of distinct isoforms of nitric oxide synthase in the juxtaglomerular apparatus of normal rat kidney. *J Am Soc Nephrol* 4, 1438-47.
- Tojo, A., Guzman, N. J., Garg, L. C., Tisher, C. C., and Madsen, K. M. (1994). Nitric oxide inhibits bafilomycin-sensitive H(+)-ATPase activity in rat cortical collecting duct. *Am J Physiol* 267, F509-15.
- Tojo, A., Madsen, K. M., and Wilcox, C. S. (1995). Expression of immunoreactive nitric oxide synthase isoforms in rat kidney. Effects of dietary salt and losartan. *Jpn Heart J* 36, 389-98.

Tojo, A., Welch, W. J., Bremer, V., Kimoto, M., Kimura, K., Omata, M., Ogawa, T., Vallance, P., and Wilcox, C. S. (1997). Colocalization of demethylating enzymes and NOS and functional effects of methylarginines in rat kidney. *Kidney Int* 52, 1593-601.

Tolins, J. P., and Shultz, P. J. (1994). Endogenous nitric oxide synthesis determines sensitivity to the pressor effect of salt. *Kidney Int* 46, 230-6.

Topper, J. N., Cai, J., Falb, D., and Gimbrone, M. A., Jr. (1996). Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A* 93, 10417-22.

Tracey, W. R., Pollock, J. S., Murad, F., Nakane, M., and Forstermann, U. (1994). Identification of an endothelial-like type III NO synthase in LLC-PK1 kidney epithelial cells. *Am J Physiol* 266, C22-8.

Traylor, T. G., and Sharma, V. S. (1992). Why NO? *Biochemistry* 31, 2847-9.

Ujiie, K., Yuen, J., Hogarth, L., Danziger, R., and Star, R. A. (1994). Localization and regulation of endothelial NO synthase mRNA expression in rat kidney. *Am J Physiol* 267, F296-302.

Vallance, P., Leone, A., Calver, A., Collier, J., and Moncada, S. (1992). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 339, 572-5.

Vaziri, N. D., Ni, Z., Wang, X. Q., Oveysi, F., and Zhou, X. J. (1998). Downregulation of nitric oxide synthase in chronic renal insufficiency: role of excess PTH. *Am J Physiol* 274 (*Renal Physiol.* 43), F642-9.

Venema, R. C., Nishida, K., Alexander, R. W., Harrison, D. G., and Murphy, T. J. (1994). Organization of the bovine gene encoding the endothelial nitric oxide synthase. *Biochim Biophys Acta* 1218, 413-20.

Venema, R. C., Sayegh, H. S., Kent, J. D., and Harrison, D. G. (1996). Identification, characterization, and comparison of the calmodulin-binding domains of the endothelial and inducible nitric oxide synthases. *J Biol Chem* 271, 6435-40.

Venema, V. J., Ju, H., Zou, R., and Venema, R. C. (1997). Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. *J Biol Chem* 272, 28187-90.

Vinay, P., Gougoux, A., and Lemieux, G. (1981). Isolation of a pure suspension of rat proximal tubules. *Am J Physiol* 241, F403-11.

Volk, K. A., Sigmund, R. D., Snyder, P. M., McDonald, F. J., Welsh, M. J., and Stokes, J. B. (1995). rENaC is the predominant Na<sup>+</sup> channel in the apical membrane of the rat renal inner medullary collecting duct. *J Clin Invest* 96, 2748-57.

von Knethen, A., Callsen, D., and Brune, B. (1999). NF-kappaB and AP-1 activation by nitric oxide attenuated apoptotic cell death in RAW 264.7 macrophages. *Mol Biol Cell* 10, 361-72.

Wagner, C., Jensen, B. L., Kramer, B. K., and Kurtz, A. (1998). Control of the renal renin system by local factors. *Kidney Int Suppl* 67, S78-83.

Wagner, C., Pfeifer, A., Ruth, P., Hofmann, F., and Kurtz, A. (1998). Role of cGMP-kinase II in the control of renin secretion and renin expression. *J Clin Invest* 102, 1576-82.

- Wakabayashi, S., Shigekawa, M., and Pouyssegur, J. (1997). Molecular physiology of vertebrate Na<sup>+</sup>/H<sup>+</sup> exchangers. *Physiol Rev* 77, 51-74.
- Wang, T. (1997). Nitric oxide regulates HCO<sub>3</sub><sup>-</sup> and Na<sup>+</sup> transport by a cGMP-mediated mechanism in the kidney proximal tubule. *Am J Physiol* 272, F242-8.
- Wang, X., Lu, M., Gao, Y., Papapetropoulos, A., Sessa, W. C., and Wang, W. (1998). Neuronal nitric oxide synthase is expressed in principal cell of collecting duct. *Am J Physiol* 275, F395-9.
- Wang, Y., Goligorsky, M. S., Lin, M., Wilcox, J. N., and Marsden, P. A. (1997). A novel, testis-specific mRNA transcript encoding an NH<sub>2</sub>-terminal truncated nitric-oxide synthase. *J Biol Chem* 272, 11392-401.
- Wang, Y., Newton, D. C., and Marsden, P. A. (1999). Neuronal NOS: gene structure, mRNA diversity, and functional relevance. *Crit Rev Neurobiol* 13, 21-43.
- Wang, Y., Newton, D. C., Robb, G. B., Kau, C. L., Miller, T. L., Cheung, A. H., Hall, A. V., VanDamme, S., Wilcox, J. N., and Marsden, P. A. (1999). RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. *Proc Natl Acad Sci U S A* 96, 12150-12155.
- Wedel, B., Humbert, P., Harteneck, C., Foerster, J., Malkewitz, J., Bohme, E., Schultz, G., and Koesling, D. (1994). Mutation of His-105 in the beta 1 subunit yields a nitric oxide- insensitive form of soluble guanylyl cyclase. *Proc Natl Acad Sci U S A* 91, 2592-6.
- Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., Xu, D., Muller, W., Moncada, S., and Liew, F. Y. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375, 408-11.

Weiss, G., Goossen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmayer, G., Wachter, H., and Hentze, M. W. (1993). Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. *Embo J* 12, 3651-7.

White, M. F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochim Biophys Acta* 822, 355-74.

Wilcox, C. S., Welch, W. J., Murad, F., Gross, S. S., Taylor, G., Levi, R., and Schmidt, H. H. (1992). Nitric oxide synthase in macula densa regulates glomerular capillary pressure. *Proc Natl Acad Sci U S A* 89, 11993-7.

Wink, D. A., Hanbauer, I., Krishna, M. C., DeGraff, W., Gamson, J., and Mitchell, J. B. (1993). Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc Natl Acad Sci U S A* 90, 9813-7.

Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S., and et al. (1991). DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 254, 1001-3.

Wolf, G., Ziyadeh, F. N., Schroeder, R., and Stahl, R. A. (1997). Angiotensin II inhibits inducible nitric oxide synthase in tubular MCT cells by a posttranscriptional mechanism. *J Am Soc Nephrol* 8, 551-7.

Wolff, D. J., Datto, G. A., and Samatovicz, R. A. (1993). The dual mode of inhibition of calmodulin-dependent nitric-oxide synthase by antifungal imidazole agents. *J Biol Chem* 268, 9430-6.

Wong, S. K., and Garbers, D. L. (1992). Receptor guanylyl cyclases. *J Clin Invest* 90, 299-305.

- Wu, F., Park, F., Cowley, A. W., Jr., and Mattson, D. L. (1999). Quantification of nitric oxide synthase activity in microdissected segments of the rat kidney. *Am J Physiol* 276, F874-F881.
- Xie, J., Roddy, P., Rife, T. K., Murad, F., and Young, A. P. (1995). Two closely linked but separable promoters for human neuronal nitric oxide synthase gene transcription. *Proc Natl Acad Sci U S A* 92, 1242-6.
- Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., and Nathan, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256, 225-8.
- Xu, J. C., Lytle, C., Zhu, T. T., Payne, J. A., Benz, E., Jr., and Forbush, B., 3rd (1994). Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. *Proc Natl Acad Sci U S A* 91, 2201-5.
- Yoshida, Y., Fogo, A., and Ichikawa, I. (1989). Glomerular hemodynamic changes vs. hypertrophy in experimental glomerular sclerosis. *Kidney Int* 35, 654-60.
- Zagotta, W. N., and Siegelbaum, S. A. (1996). Structure and function of cyclic nucleotide-gated channels. *Annu Rev Neurosci* 19, 235-63.
- Zatz, R., and de Nucci, G. (1991). Effects of acute nitric oxide inhibition on rat glomerular microcirculation. *Am J Physiol* 261, F360-3.
- Zeidel, M. L., Seifter, J. L., Lear, S., Brenner, B. M., and Silva, P. (1986). Atrial peptides inhibit oxygen consumption in kidney medullary collecting duct cells. *Am J Physiol* 251, F379-83.

Zhang, J., and Snyder, S. H. (1992). Nitric oxide stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Proc Natl Acad Sci U S A* 89, 9382-5.

Zizak, M., Lamprecht, G., Steplock, D., Tariq, N., Shenolikar, S., Donowitz, M., Yun, C. H., and Weinman, E. J. (1999). cAMP-induced phosphorylation and inhibition of Na(+)/H(+) exchanger 3 (NHE3) are dependent on the presence but not the phosphorylation of NHE regulatory factor. *J Biol Chem* 274, 24753-8.

Zou, A. P., and Cowley, A. W., Jr. (1997). Nitric oxide in renal cortex and medulla. An in vivo microdialysis study. *Hypertension* 29, 194-8.

Zou, A. P., Wu, F., and Cowley, A. W., Jr. (1998). Protective effect of angiotensin II-induced increase in nitric oxide in the renal medullary circulation. *Hypertension* 31, 271-6.

## CHAPTER 8: BIBLIOGRAPHY

### **Awards and Scholarships**

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#### **External Awards:**

2000-2003: Medical Research Council of Canada award

1998 -1999: Ontario Graduate Scholarship

1996 - 1998: Natural Sciences and Engineering Research Council of Canada award

1995 - 1996: Ontario Graduate Scholarship

1995 (declined): Canadian Hypertension Society/Pfizer/MRC Graduate Studentship

1993: Kidney Foundation of Canada Summer Studentship

#### **University Awards:**

May 1998: The Gerry Taichman award

1995 - 1999: University of Ottawa Excellence Scholarship

#### **Other**

Sept 1998: Canadian Society of Nephrology Trainee award

Sept 1995: Canadian Society of Nephrology Trainee award

## Publications

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### Peer-Reviewed Articles

- 1) Roczniak, A. and K. D. Burns. Nitric oxide (NO) inhibits p27<sup>kip1</sup> expression in rabbit proximal tubule (PT) cells. In preparation.
- 2) Roczniak, A., D. Z. Levine, and K. D. Burns. Localization of protein inhibitor of neuronal nitric oxide synthase (PIN) in rat kidney. Submitted to Am. J. Physiol.
- 3) Roczniak, A., J. N. Fryer, D. Z. Levine, and K. D. Burns. Downregulation of neuronal nitric oxide synthase in the rat remnant kidney. J. Am. Soc. Nephrol. 10: 704 - 713, 1999.
- 4) Roczniak, A., J. Zimpelmann, and K. D. Burns. Effect of dietary salt on neuronal nitric oxide synthase in the inner medullary collecting duct. Am. J. Physiol. 275 (Renal Physiol. 44): F46 - F54, 1998.
- 5) Roczniak, A., and K. D. Burns. Nitric oxide stimulates guanylate cyclase and regulates sodium transport in rabbit proximal tubule. Am. J. Physiol. 270 (Renal Fluid Electrolyte Physiol. 39): F106- F115, 1996.
- 6) Burns, K. D., L. Regnier., A. Roczniak., and R. L. Hebert. Immortalized rabbit cortical collecting duct cells express AT<sub>1</sub> angiotensin II receptors. Am. J. Physiol. 271 (Renal Fluid Electrolyte Physiol. 40): F1147- F1157, 1996.

### Abstracts and presentations (all peer-reviewed)

- 1) Roczniak, A. and K. D. Burns. Nitric oxide (NO) inhibits p27<sup>kip1</sup> expression in rabbit proximal tubule (PT) cells. J. Am. Soc. Nephrol. 10:1999.  
**Poster presented by A. Roczniak at the Annual Meeting of the American Society of Nephrology, Miami, Florida. Nov. 1999.**
- 2) Roczniak, A. and K. D. Burns. Localization and regulation of protein inhibitor of neuronal nitric oxide synthase (PIN) in rat kidney. J. Am. Soc. Nephrol. 10: 1999.  
**Poster presented by A. Roczniak at the Annual Meeting of the American Society of Nephrology, Miami, Florida. Nov. 1999.**
- 3) Roczniak, A., D. Z. Levine, J. Fryer, and K. D. Burns. Effect of losartan on intrarenal neuronal nitric oxide synthase in the 5/6 nephrectomized rat. J. Am. Soc. Nephrol. 9: 621, 1998.  
**Poster presented by A. Roczniak at the Annual Meeting of the American Society of Nephrology, Philadelphia, Pennsylvania. Oct. 1998.**

- 4) Roczniak, A., D. Z. Levine, J. Fryer, and K. D. Burns. Downregulation of intrarenal neuronal nitric oxide synthase in the 5/6 nephrectomized rat: role of angiotensin II AT1 receptors. Clin. Invest. Med. S61, 1998.  
**Oral presentation by A. Roczniak at the Royal College of Physicians and Surgeons of Canada Annual Meeting, Toronto, Ontario, September 1998.**
- 5) Roczniak, A., D. Z. Levine, J. Fryer, and K. D. Burns. Downregulation of intrarenal neuronal nitric oxide synthase in the 5/6 nephrectomized rat: role of Angiotensin II AT<sub>1</sub> receptors.  
**Oral presentation by A. Roczniak at the Ontario Chapter of the Canadian Hypertension Association Spring Meeting, Kingston, Ontario, May 1998.**
- 6) Roczniak, A., J. Zimpelmann, D. Z. Levine, and K. D. Burns. Intrarenal expression of neuronal nitric oxide synthase (nNOS) is down-regulated in the 5/6 nephrectomized (Nx) rat. J. Am. Soc. Nephrol. 8: 628, 1997.  
**Poster presented by A. Roczniak at the Annual Meeting of the American Society of Nephrology, San Antonio, Texas. Nov. 1997.**
- 7) Roczniak, A., and K. D. Burns. Effect of dietary NaCl on inducible nitric oxide synthase (iNOS) expression in rat inner medullary collecting duct (IMCD). J. Am. Soc. Nephrol. 7: 1541, 1996.  
**Poster presented by A. Roczniak at the Annual Meeting of the American Society of Nephrology, New Orleans, Louisiana. Nov. 1996.**
- 8) Burns, K. D., Y-H. Wang, J. Kontogiannis, G. Wehbi, and A. Roczniak. Nitric oxide (NO) stimulates growth of proximal tubule (PT) cells. J. Am. Soc. Nephrol. 6: 764, 1995.  
**Poster presented by A. Roczniak at the Annual Meeting of the American Society of Nephrology, San Diego, Cal. Nov. 1995.**
- 9) Roczniak, A., and K. D. Burns. Nitric oxide (NO) regulates sodium transport in rabbit proximal tubule. Clin. Invest. Med. 18: B93, 1995.  
**Oral presentation by A. Roczniak at the Annual Meeting of CSCI/RCPSC, Montreal, Sept. 1995.**  
**Recipient of the Canadian Society of Nephrology Trainee award.**
- 10) Burns, K. D., L. Regnier., A. Roczniak, D. Lajeunesse, and R. L. Hebert. An immortalized rabbit cortical collecting duct cell line with functional angiotensin II receptors. J. Am. Soc. Nephrol. 5: 656, 1994.
- 11) Roczniak, A., and K. D. Burns. Exogenous nitrates increase cyclic GMP (cGMP) and inhibit Na<sup>+</sup>/H<sup>+</sup> exchange in rabbit proximal tubule (PT). J. Am. Soc. Nephrol. 5: 259, 1994.  
**Poster presented by A. Roczniak at the Annual Meeting of the American Society of Nephrology, Orlando, Fla. Oct. 1994.**

- 12) Burns, K. D., L. Regnier, A. Rocznik, D. Lajeunesse, and R. L. Hebert. Creation of an immortalized rabbit cortical collecting duct cell line that expresses angiotensin II receptors. *Clin. Invest. Med.* 17: 535, 1994