Recent Advances in the Regulation of Nitric Oxide in the Kidney

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Abstract—Nitric oxide (NO) plays important roles in the regulation of renal function and the long-term control of blood pressure. New roles of NO have been proposed recently in diabetes, nephrotoxicity, and pregnancy. NO derived from all 3 NOS isoforms contributes to the overall regulation of kidney function, and recent advances in our understanding of their regulation have been made lately. In this regard, substrate and cofactor availability play important roles in regulating nitric oxide synthase (NOS) activity not only by limiting enzyme activity but also by influencing the coupling of NOS with its cofactors, tetrahydrobiopterin and NADPH. Protein–protein interactions are now recognized to be important negative and positive regulators of NOS. Phosphorylation is another component of the mechanism whereby NOS is activated or deactivated. Increased NOS expression can also influence enzyme activity; however, the degree of expression does not always correlate with enzyme activity because increased NO levels can result in inhibition of NOS. Finally, other potential regulators of NOS such as endogenous L-arginine analogs may also be important. In this article, we summarize recent advances in the regulation of activity and expression of the NOS isoforms within the kidney. (Hypertension. 2005;45:1062-1067.)

Key Words: hemodynamics ■ nitric oxide ■ nitric oxide synthase ■ sodium

Nitric oxide (NO) plays an important role in the control of renal function and long-term regulation of blood pressure.1-4 This is best evidenced by the fact that inhibiting intrarenal NO production increased blood pressure.5 In addition, reduced NO has been identified as a common denominator of many hypertensive models.6-9 The effects of NO on blood pressure via actions in the kidney occur through multiple mechanisms. These include increasing renal blood flow caused by vasodilatation,10 increasing glomerular filtration,11 inhibiting sodium transport along the nephron,12-14 and regulating release of renin.15 NO produced by each of the 3 nitric oxide syntheses (NOS), NOS 1, NOS 2, and NOS 3, reportedly contributes to the regulation of renal function. Inhibition of NOS activity within the kidney is known to lead to sodium retention and hypertension. This review addresses recent advances in our understanding of the role played by renal NO in various physiological and pathophysiological conditions, as well as how NO production is regulated.

Roles for Renal NO

Historically, NO produced by the kidney has been thought of primarily as a factor that regulates urinary volume and sodium excretion. The physiological effects of NO can be mediated via changes in renal hemodynamics and/or salt and water absorption by the nephron. NO reduces renal vascular tone in part by dilating the afferent arteriole.16 It also increases glomerular filtration rate.11 NO modulates renin secretion by the juxtaglomerular apparatus15 and tubuloglomerular feedback.3 Finally, NO regulates transport in various nephron segments as reviewed recently by Ortiz and Garvin.12

More recently it has been recognized that in a number of pathophysiological conditions, the actions of NO on renal hemodynamics and/or nephron transport are altered. In early diabetes, NO appears to play a more pronounced role in the maintenance of blood pressure. NO inhibition results in hypertension in diabetic but not in control rats,17 showing that acute NOS 1 inhibition reduces glomerular filtration rate only in diabetic rats, and therefore suggesting that NO plays an enhanced role in regulating kidney function during diabetes.18 NO also plays a protective role within the kidney. Augmenting NO by means of NO donors decreases nephrotoxicity caused by acetaminophen.19 NOS 3 polymorphisms associated with decreased NO production correlate with end-stage renal disease in humans.20 These data suggest that lack of renal NO may be important in advanced nephropathy and renal damage.21 In addition, promoting NO production by administering L-arginine is known to attenuate pregnancy-induced hypertension.22 This may be caused by effects on the kidney, because large increases in NO that enhance renal blood flow occur during normal pregnancy.23-26 However, the precise role of each of the 3 NOS isoforms is unclear in these pathological as well as physiological circumstances.
The use of selective NOS inhibitors and knockout mice has allowed us in some instances to investigate the individual roles of the 3 NOS isoforms in regulating renal function. However, we have not made a great deal of progress in identifying the role of NO produced by a given isoform in a given cell type. This is caused by a variety of factors, including: (1) the multiplicity of NOS isoforms, which are activated by different stimuli; (2) varying expression of NOS isoforms in different cell types; (3) differences in chronic regulation of expression and activity of the various NOS isoforms; and (4) the complex anatomy of the kidney, such that NO produced in one cell type can act in a different cell type.

As a first step in addressing this problem, our laboratory developed a technique to restore the function of a single NOS isoform in a single nephron segment, as in endothelial cells.27 NOS 3 is responsible for autocrine inhibition of NaCl absorption in the thick ascending limb.28 We showed that NOS 3 function could be restored selectively to this segment of NOS 3 knockout mice using an adenovirus with a tissue-specific promoter.13 Because we were able to transduce \( \approx 75\% \) to 95% of thick ascending limbs in vivo, we are now studying the specific role of the medullary thick ascending limb NOS 3 in the regulation of salt and water excretion in vivo. In theory, this same approach could be used for the other NOS isoforms in other cell types, provided that one has the appropriate tissue-specific promoter.

### Regulation of NOS Activity

NOS 129 and NOS 330 have been thought to be regulated primarily by increases in intracellular calcium and NOS 2 by changes in expression of the enzyme.31 However, these views have recently been challenged by studies in a number of renal cells. Changes in substrate and cofactor availability, protein–protein interactions and phosphorylation state have all come to light as significant regulators of NOS activity. Availability of substrate and cofactors as a limiting factor of NOS activity has been primarily attributed to pathophysiological situations. However, cofactor availability may also be a physiological regulator of NOS activity. Increasing NaCl concentration in the lumen of the macula densa activates NOS 129 and also raises intracellular pH. Wang et al.32 first reported that the increase in intracellular pH caused by increasing luminal NaCl may activate macula densa NOS 1. Blocking luminal Na+/H+ exchange (which prevents alkalization of macula densa cells) augmented tubuloglomerular feedback similarly to selective NOS 1 inhibition. More recently, Liu et al.33 found that the increase in NO production caused by elevated NaCl was blunted when the increase in intracellular pH was blocked, and that raising intracellular pH without increasing luminal NaCl was sufficient to induce NO production. A similar effect of pH on NOS 2 activity was also reported in mesangial cells.34 In these cells, reducing extracellular pH, and presumably intracellular pH, decreased NOS 2 activity by 80%.

The mechanism by which pH alters NOS 1 and 2 activity appears to involve a combination of direct effects of protons per se on the enzyme and availability of the cofactor NADPH. Decreased NOS 1 activity at low pH has been shown to be caused by “uncoupling” of NADPH oxidation, resulting in increased formation of \( \text{H}_2\text{O}_2 \).35 Mesangial cells exposed to low pH showed an increase in oxidized nicotinamide adenine dinucleotide phosphate/citrate ratio.34 The authors concluded that at low intracellular pH, there is less NADPH to accept electrons from NOS 2 during production of NO, so that NADPH is “uncoupled” from NO production. Interestingly, when this occurs, NOS would be predicted to produce superoxide, which could scavenge any NO produced. However, at present we know of no reports regarding NOS uncoupling in other structures within the kidney, such as vasculature and nephron segments.

The ability of changes in pH to regulate NOS 3 has not been investigated to our knowledge. However, it would be surprising if it did not control NO production by NOS 3 because this parameter modulates both NOS 1 and NOS 2 activity. Because virtually all cells have transporters to regulate intracellular pH, this mechanism may play an important role in all renal cells. Furthermore, regulation of NOS activity by intracellular pH may link NO production with acid/base balance and superoxide generation. The latter has recently been shown to depend on Na+/H+ exchange activity in the thick ascending limb.36

In addition to NADPH, the availability of tetrahydrobiop terin and arginine may also control NOS activity. A decrease in the ratio of reduced tetrahydrobiop terin to oxidized dihydrobiop terin in the renal medulla has been shown to blunt NO production, and has been proposed to contribute to salt-sensitive hypertension.37 Oral \( \text{L} \)-arginine supplementation reverses p47 phox and gp91 phox expression induced by high salt in the renal cortex of Dahl rats,38 suggesting that substrate supplementation can restore the imbalance between NO and reactive oxygen species, presumably by increasing NOS-derived NO. In addition, \( \text{L} \)-arginine transport has been shown to affect NOS activity and NO production in the renal medulla.39 Arginine transport by the \( \text{y}^+ \) transporter may be especially significant in angiotensin-dependent forms of hypertension, because \( \text{y}^+ \) activity and expression are regulated by angiotensin.40

Activity of all 3 NOS isoforms is modulated by protein–protein interactions.41 Protein inhibitor of neuronal NOS (PIN),42,43 caveolin-1,44 caveolin-3,45 and several proteins bearing PDZ domains46 that influence targeting to discrete membrane domains of excitable tissues regulate NOS 1 activity. Although some of these proteins have been localized to the kidney,47 we know of no studies investigating the role of these proteins in renal NOS 1 activity.

Several proteins have been identified that directly interact with NOS 2. Kalirin and NOS-associated protein-110 have been shown to interact with NOS 2 and inhibit its activity.48 In addition, Kuncewicz et al.49 found that Rac1 and Rac2, members of the Rho GTPase family, both interact with NOS 2. These authors also demonstrated that the point of interaction for Rac2 is the NOS 2 oxygenase domain and that overexpression of Rac2 augments NO production in immune-activated murine macrophages. Because Rac is important for assembly and activation of NADPH oxidase, this finding suggests coordinated regulation of NADPH oxidase and NOS...
2-derived NO production. However, these interactions have not been shown to occur in renal cells to our knowledge.

Since the original publications showing that NOS 3 activity is inhibited by caveolin-1 and enhanced by heat shock protein 90 in endothelial cells, several other protein–protein interactions have been defined, including discovery of the NOS 3 inhibitory proteins NOSIP and NOSTRIN. It is likely that these regulatory proteins alter NOS 3 activity in all endothelial cells, including those in the kidney. However, their significance in the regulation of NOS 3 activity and/or expression in renal epithelial and interstitial cells has not been thoroughly investigated except for the interaction of NOS 3 and heat shock protein 90. Recently, activation and translocation of NOS 3 in the thick ascending limb have been reported to require heat shock protein 90 ATPase activity.

Phosphorylation of NOS 3 by protein kinase A was first reported in 2001. However, not until NOS 3 activity by shear stress was shown to be mediated by phosphorylation of serine 1179 in endothelial cells were the potential consequences appreciated. Flow-induced activation of NO production occurs in afferent arterioles, inner medullary collecting ducts, and thick ascending limbs. In thick ascending limbs, activation of NOS 3 by flow is caused by phosphorylation of serine 1179, as it is in endothelial cells. It is unclear whether this is also true for renal vessels, because flow-induced activation of NOS 3 in the vasa recta does not appear to be caused by phosphorylation at serine 1179, but rather simply an increase in intracellular calcium. In addition to serine 1179, at least 4 other phosphorylation sites on NOS 3 are known. Insulin has also been suggested to enhance NOS 3 activity in the renal medulla by dephosphorylating threonine 495 in diabetic rats. Phosphorylation of NOS 3 at this amino acid may be significant because threonine 495 has been proposed to be a “switch” that determines whether NOS 3 produces NO or superoxide. Thus, measurements of bioavailable NO are crucial to determine the physiological significance of increased NOS phosphorylation at this amino acid.

Compared with our understanding of the role of phosphorylation of specific amino acids in NOS 3, our knowledge of NOS 1 and 2 is minimal. NOS 1 is known to be constitutively phosphorylated at serine 741, and dephosphorylation at this amino acid increases enzyme activity. In addition, phosphorylation at serine 847 can attenuate the catalytic activity of the enzyme in neuronal cells. Presumably, homologous amino acids in the different NOS isoforms have the potential to be phosphorylated; however, the effect of phosphorylating such putative sites on NOS 1 and NOS 2 within the kidney has not been studied to our knowledge.

**Hormonal Regulation of NOS**

Renal NOS activity has been shown to be acutely regulated by several humoral factors. Arima et al. reported that aldosterone can activate NOS 3 in renal arterioles, leading to NO production; however, the mechanism involved was not investigated. Taylor et al. found that in ET receptor-deficient rats, medullary NOS activity and renal endothelin production are decreased, indicating that renal endothelin regulates NOS 3 activity as reported for the thick ascending limb. Mori et al. found that vasopressin stimulated a rapid increase in intracellular NO via increased intracellular calcium levels in the inner medullary collecting duct. Although they did not assess which NOS isoform was activated, NOS 3 is likely responsible because of its high expression in the collecting duct. Finally, angiotensin II acutely regulates NO production by NOS 1 in the macula densa, apparently because of increased intracellular calcium. However, the physiological significance of this finding is unclear, because angiotensin II enhances tubuloglomerular feedback, whereas NO inhibits it.

**Regulation of Expression**

Chronic changes in NOS expression may be important in conditions such as high salt intake and diabetes. High salt increases the expression of all 3 NOS isoforms in the medulla. The mechanisms by which this occurs have not been worked out for NOS 1 and 2, but have been defined for NOS 3 in medullary thick ascending limbs. High salt increased outer medullary osmolality and hyperosmolality enhanced NOS 3 expression in primary thick ascending limb cultures, and an ET receptor antagonist blocked this effect. Hyperosmolality also enhanced endothelin-1 release. Finally, in vivo a dual ET/ET antagonist blocked the effects of high salt on NOS 3 expression. In addition, a low-sodium diet causes chaperone heat shock protein 90 to relocate from the apical to the basolateral side of the thick ascending limb. Because NOS 3 is known to interact with heat shock protein 90, leading to increased enzyme activity, heat shock protein 90 may play a role in regulation of NOS 3 expression by salt intake.

Exposure to endothelin-1 alone also augments NOS 3 expression in the thick ascending limb. Similarly, endothelin-1 stimulates NOS 3 expression in inner medullary collecting ducts. However, unlike the thick ascending limb where the effect was mediated only by ET receptors, in the inner medullary collecting duct both ET and ET were involved. Osmotic stimuli have also been shown to increase NOS 3 expression in inner medullary collecting ducts in culture. This may be important for regulation of function in the renal medulla, where osmolality is extremely variable and dependent on salt and water intake. Given that similar factors regulate NOS 3 expression in the inner medullary collecting duct and thick ascending limb, high salt may induce expression by similar mechanisms in both cell types.

Changes in NOS expression may also be important in diabetes. High glucose increases NOS 2 mRNA and protein expression in mesangial cells in the presence of cytokines. The increase was mediated by protein kinase C. Transcriptional/translational regulation of NOS 2 in mesangial cells by glucose may also involve JAK2, p38 MAPK, and nuclear factor κB, which have been shown to regulate NOS 2 expression in renal epithelial cells.

Repression of NOS 2 transcription may be just as important as induction in controlling the final amount of NOS 2 protein. Yu and Kone demonstrated that treating mesangial cells with DNA methylation inhibitors augmented cytokine induction of endogenous NO production and NOS 2 protein. In vitro methylation of the NOS 2 promoter blunted its
activity, whereas inhibition of DNA methyltransferase increased NOS 2 promoter activity and nitrate production. Moreover, in vitro methylation inhibited binding of nuclear factor κB to the NOS 2 enhancer element. These results suggest that cytosine methylation is an important repressor of NOS 2 transcription in these cells. Whether NOS 1 and 3 expressions are regulated in diabetes is unclear and the mechanisms involved have not been extensively studied.

Other Regulators of NOS Activity

In addition to the regulators described, several other compounds may modulate NOS activity in the kidney. The most important of these in terms of hypertension may be the endogenous l-arginine analogues such as asymmetrical dimethylarginine. This compound inhibits NOS activity, and circulating concentrations are elevated in hypertension and by high salt intake. Carbon monoxide produced by heme oxygenase has recently been recognized as a NOS regulator, although the nature of this interaction is still unclear. Several drugs may have a marked impact on NO production. Dobrian et al recently reported that the peroxisome proliferator-activated receptor-γ agonist and insulin sensitizer pioglitazone prevented the development of hypertension in obese hypertensive rats. This therapeutic effect was at least partially attributed to increased renal NO production and bioavailability caused by decreased superoxide generation. However, the exact site of NO production is unknown, because PPAR-γ receptors are present in many renal cells that express NOS and produce NO. Finally, reactive oxygen species production by the kidney and their role in scavenging NO have recently received a great deal of attention. Superoxide plays a quintessential role in determining NO bioavailability and thus its effect. This topic is beyond the scope of this review and has recently been reviewed.

Perspectives

The complexity of the kidney, with its nearly 20 different tissue types arranged in a geometry that also impacts on function, has slowed progress of both cellular and whole-animal approaches to understanding the role of NO in regulating renal function. To study the contribution of the various NOS isoforms, 2 lines of research have been followed: (1) assessment of renal function using isolated cells and individual segments; and (2) studies of renal function in vivo. Part of our limitation in understanding NOS regulation comes from our inability to measure biologically active NO in intact systems. This is important because: (1) protein expression does not necessarily correlate with enzyme activity and thus NO production; (2) the assay normally used to assess enzyme activity (conversion of l-arginine to l-citrulline) requires the addition of substrate and cofactors, and thus regulation of enzyme activity by decreased substrate or cofactor availability may be missed; and (3) enzyme activity may not represent bioavailable NO, because NO can rapidly be scavenged by other substances such as superoxide. Although much progress has been made regarding our understanding of NOS regulation within the kidney, in some instances the information was obtained from nonrenal cells and in vitro systems, and thus more research is required to fully understand the mechanisms whereby NOS is regulated within the different structures in the kidney as well as their physiological significance. New technology that allows deletion or expression of a particular gene in specific tissues in the kidney is an important scientific advance. Over the past few years, various animal models have been developed using such technology that provide new means of studying the physiological actions of a single NOS isoform in a single cell type at a specific point in time in vivo.

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