Paradoxes of nitric oxide in the diabetic kidney

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Komers, Radko and Sharon Anderson. Paradoxes of nitric oxide in the diabetic kidney. Am J Physiol Renal Physiol 284: F1121–F1137, 2003; 10.1152/ajprenal.00265.2002.—As an important modulator of renal function and morphology, the nitric oxide (NO) system has been extensively studied in the diabetic kidney. However, a number of studies in different experimental and clinical settings have produced often confusing data and contradictory findings. We have reviewed a wide spectrum of findings and issues that have amassed concerning the pathophysiology of the renal NO system in diabetes, pointed out the controversies, and attempted to find some explanation for these discrepancies. Severe diabetes with profound insulinopenia can be viewed as a state of generalized NO deficiency, including in the kidney. However, we have focused our hypotheses and conclusions on the events occurring during moderate glycemic control with some degree of treatment with exogenous insulin, representing more the clinically applicable state of diabetic nephropathy. Available evidence suggests that diabetes triggers mechanisms that in parallel enhance and suppress NO bioavailability in the kidney. We hypothesize that during the early phases of nephropathy, the balance between these two opposing forces is shifted toward NO. This plays a role in the development of characteristic hemodynamic changes and may contribute to consequent structural alterations in glomeruli. Both endothelial (eNOS) and neuronal NO synthase can contribute to altered NO production. These enzymes, particularly eNOS, can be activated by Ca2+/calmodulin-independent and alternative routes of activation that may be elusive in traditional methods of investigation. As the duration of exposure to the diabetic milieu increases, factors that suppress NO bioavailability gradually prevail. Increasing accumulations of advanced glycation end products may be one of the culprits in this process. In addition, this balance is continuously modified by actual metabolic control and the degree of insulinopenia.

reactive oxygen species; endothelial function; renal function; signal transduction; diabetic nephropathy

NITRIC OXIDE (NO) IS A PARACRINE mediator with a wide spectrum of physiological actions, including the control of vascular tone, antithrombotic actions, cell cycle regulation, neurotransmission, signal transduction, and inflammation. NO is synthesized during conversion of its physiological precursor L-arginine (L-Arg) to L-citrulline (77). This reaction is catalyzed by a family of enzymes known as NO synthases (NOS) (81). Three NOS isoforms [neuronal (nNOS, NOS1); inducible (iNOS, NOS2); and endothelial (eNOS, NOS3)] have been identified in mammalian tissues. nNOS and eNOS are traditionally viewed as constitutive enzymes, with a limited tissue distribution, exhibiting intracellular Ca2+/calmodulin dependency, although Ca2+-independent activation has been described (29, 34). nNOS and eNOS are responsible for producing NO for a variety of physiological purposes (68). In contrast, iNOS is an inducible enzyme, expressed in all nucleated cells that generate large bursts of NO in response to immunological and certain nonimmunological stimuli. iNOS is usually described as Ca2+ independent, although the presence of Ca2+ enhances iNOS activity (142). To produce NO, NOS enzymes require molecular oxygen and a battery of cofactors and post-translational modifications (39, 68). Effects of NO are typically mediated by activation of soluble guanylate cyclase, resulting in increased levels of cGMP (85). NO also acts as a potent modulator of renal function. A wealth of information on renal actions of NO that has

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amassed during the past two decades has been summarized in several excellent reviews (9, 68). All three NOS isoforms are found in the kidney. nNOS protein and mRNA are found predominantly in the macula densa (MD) region of the distal tubule and renal nerves (7, 64, 127, 150). iNOS mRNA is detectable in most of tubular cells along the nephron (1, 84). eNOS is typically expressed in endothelial cells along the renal vascular tree (7). All three isoforms are expressed in the medulla (88, 104, 123), and medullary NO production exceeds that in the cortex (154). Studies with nonspecific NOS inhibitors, such as L-Arg analogs, demonstrate that renal hemodynamics are very sensitive to NOS inhibition. NO controls both afferent and efferent vascular tone, the ultrafiltration coefficient (28, 159), and medullary blood flow (79), with preferential action on the afferent arteriole (28). In addition, NO has natriuretic actions (4, 79).

As an important modulator of renal function and morphology, the NO system has been extensively studied in the diabetic kidney. However, a number of studies in different experimental settings have often produced confusing data and contradictory findings. In this paper, we will briefly review in vitro evidence on the renal NO system in diabetes; put more emphasis on in vivo evidence, in particular, pointing out the controversies in this literature; and attempt to find some explanation for these discrepancies. Furthermore, based on prevailing evidence, we will propose a unifying hypothesis on the changes in renal NO in diabetes and its role in the pathophysiology of nephropathy.

EFFECTS OF DIABETES ON THE RENAL NO SYSTEM IN VITRO

Determinations of the effects of high glucose or glycosylation products on NO bioavailability in renal cell cultures (143) and in isolated glomeruli ex vivo (22, 23, 112, 146) have been applied by several groups as an in vitro approach to assess the effects of diabetes on the renal NO system. Considering the abundance of endothelial cells in the kidney, we also mention several reports on this cell type. Although not performed directly in renal cells, we assume that the same processes as described by these authors could be active in the kidney and should be discussed in the context of this review (13, 30, 46). In general, in vitro studies have provided important information on the mechanisms whereby high glucose or glycosylation products influence NO bioavailability. These mechanisms include enhanced synthesis or action of the prostanoid thromboxane A2 and PKC activation (22, 23, 46), NO quenching (14, 143), inhibition of both Ca++-dependent and -independent NOS activities (30, 143), reactive oxygen species (ROS) production (46), and NO capture by glucose (13). Taken together, these studies have suggested a decrease in renal NO production, action, stability, or bioavailability in diabetes. In fact, in vitro studies have provided most of the experimentally available evidence implicating diabetes as a state of renal NO deficiency.

Unlike the previously mentioned approaches, in vitro measurements of NO-dependent renal vascular reactivity also provide functional aspects of the renal NO system and are therefore discussed in more detail. Evidence discussed in this section encompasses studies using various experimental approaches and models, including the isolated kidney and isolated arteriolar and renal artery preparations (Table 1).

Studies in isolated rat kidneys reported increased (11) or normal (10) vasodilator responses to ACh or metacholine and normal responses to nitroprusside (10, 11). However, other investigators have reported convincing evidence suggesting deleterious effects of diabetes on renal NO-dependent vascular reactivity. Ohishi and Carmines (90) studied isolated kidney preparations using videomicroscopy of in vitro blood-perfused juxtaglomerular nephrons to directly assess renal afferent and efferent arteriolar diameters. Diabetic kidneys demonstrated greater baseline afferent diameters, whereas efferent diameters did not differ between diabetic and control rats. Apparently, this model reflects changes in arteriolar diameters in diabetic kidneys similar to those observed in vivo in micropuncture studies (50, 160). Both afferent and efferent responses to the NO inhibitor Nω-nitro-L-arginine (L-NNA) were blunted in diabetic kidneys. The responses to NOS inhibition in diabetic rats were restored by treatment with SOD. These observations suggested decreased NO availability in diabetic glomerular arterioles, possibly due to accumulation of NO-scavenging superoxide anions. Interestingly, this NO-deficient situation was observed in kidneys with decreased afferent arteriolar tone. Corresponding observations have been reported in alloxan-diabetic rabbits. ACh-induced vasodilator responses in preconstricted, microperfused afferent arterioles were impaired in diabetic rabbits, and the defect was corrected by using the SOD mimetic tempol (109). However, using a similar model, Moore et al. (86) reported somewhat different data compared with the two previously mentioned studies. The responses to ACh were unaltered in diabetic arterioles. Although the fractional Nω-nitro-L-arginine methyl ester (L-NAME)-induced decrease in afferent diameter was lower in diabetic rats, absolute changes were greater in diabetic vessels. This discrepancy was attributable to differences in baseline diameters between control and diabetic rats. Furthermore, the authors performed direct intraluminal NO measurements and observed a marked increase in NO concentrations in diabetic vessels after perfusion with a high-glucose perfusate. The authors also explored the vasoactive effects of advanced glycation end products (AGEs). The AGE-containing perfusate induced no changes in diabetic vessels, although it blunted responses to ACh. Thus these findings are only partially consistent with previously mentioned studies by Bucala et al. (14) and do not suggest a substantial role for AGE in NO quenching.

An interplay between NO and ANG II was addressed by Schoonmaker et al. (111). Control afferent arterioles had enhanced vasoconstrictor responses to ANG II
when pretreated with L-NNA. In contrast, L-NNA-treated and untreated arteries harvested from diabetic rats demonstrated no differences in ANG II-induced vasoconstriction. Moreover, responses of efferent arterioles were similar in control and diabetic rats (111). These observations corresponded to previous findings in rabbit afferent arteriolar preparations micropерfused with a normal and high-glucose (30 mM) perfusate (6) and suggested attenuated NO action in counterbalancing the effects of ANG II specifically in afferent arterioles in diabetes and/or hyperglycemia.

Diabetes-associated changes in the NO system have also been investigated in isolated renal arteries. It is likely that observations in renal arteries reflect changes in the vascular system in general rather than contribute to an understanding of specific renal processes. However, controversial findings in this area may signal some specific features of the renal arteries compared with other large-conduit vessels that usually display diabetes-induced defects in endothelium-dependent vasodilation (99). Dai et al. (25) reported that the defect in ACh-induced vasodilation in diabetic rats was ameliorated by pretreatment with a hydroxyl radical scavenger or with a blockade of prostaglandin H₂-thromboxane A₂ receptors. Corresponding to studies in other experimental settings (23, 90, 109), these data suggest an impairment of NO endothelial production or action in diabetic arteries, possibly mediated by the increased production of ROS and prostanooids that oppose the effects of NO. However, more recent studies in that model found unchanged responses to ACh in renal arteries from insulin-treated diabetic rats preconstricted with serotonin and even enhanced responses to insulin compared with controls (130, 131). In contrast to data by Dai et al. (25), enhanced responses to ACh and to NOS inhibition have also been reported in renal arteries harvested from untreated alloxan-diabetic rabbits (3).

Unlike the studies in cultured renal cells, evaluation of renal vascular reactivity in vitro has provided more controversial evidence, suggesting enhanced, normal, or decreased NO synthesis and activity in the diabetic kidney (Table 1). These contrasting findings cannot be explained by species differences, differences in duration of diabetes, metabolic control, or the presence or absence of insulin treatment.

One of the factors that could possibly explain differences among at least some of these studies could relate to the method of preconstriction of isolated vessels. There is evidence suggesting that different vasoconstrictor stimuli may alter responses to ACh in afferent arterioles (43). Different constrictors may also exert variable effects on basal vascular tone and, as in the case of serotonin, even activate eNOS (107).

Several caveats of in vitro approaches in evaluating the renal NO system in diabetes in general merit consideration. The in vitro studies may not precisely reflect the diabetic milieu, which is determined not only by high glucose. For example, most of the in vitro evidence regarding the effects of glucose on cellular NO systems has been obtained by using media glucose concentrations from 25 to 33 mM. The clinical correlate would be severely decompensated diabetes, which is known to be associated with renal vasoconstriction as a result not only of specific effects of glucose on renal cells but also of physiological compensation for volume depletion and electrolyte wasting (50). However, as specifically discussed below, early stages of nephropathy are characterized by glomerular hyperperfusion, which has important pathophysiological implications.
and is prominent in the more clinically applicable model of moderate hyperglycemia (15–20 mM) (52, 162). Consequently, it is beneficial to use an in vitro approach to precisely model a particular condition and obtain answers to specific questions. However, the in vitro data should be interpreted with caution, considering functional alterations typical in the early stages of diabetic nephropathy.

Several in vitro studies also focused on the effects of hyperglycemia on iNOS-derived NO in renal cell cultures (113). Considering the relatively weak in vivo evidence for the role of iNOS in diabetes-induced alterations in the renal NO system, these papers are not discussed in this review. Consequently, we also do not discuss in vitro studies in mesangial cells. With respect to prevailing evidence, this cell type does not express constitutive NOS (68).

**EFFECTS OF DIABETES ON THE RENAL NO SYSTEM IN VIVO**

**Expression of NOS Isoforms in the Diabetic Kidney In Vivo**

A number of studies have examined renal NOS expression in diabetes in vivo (Table 2). Some of the studies mentioned in this section combined measurements of NOS expression with renal hemodynamics and will be discussed further in the following sections. Choi et al. (19) found markedly increased renal cortical expression of all NOS isoforms in streptozotocin (STZ)-diabetic rats; medullary expression of NOS was not different from controls. Another group (118) found enhanced immunohistochemical expression and NADPH diaphorase staining, reflecting constitutive NOS activity, in endothelia of afferent but not efferent arterioles of STZ-diabetic rats. Enhanced NADPH diaphorase staining was associated with increased afferent diameter, increased glomerular filtration rate (GFR) assessed by creatinine clearance, and glomerular hypertrophy. These changes were corrected by insulin treatment or by treatment with l-NAME. In accordance with the previous report, Veelken et al. (141) reported increased cortical eNOS expression in untreated hyperfiltering diabetic rats. In addition, increased medullary expressions of nNOS and eNOS were found in the medulla of diabetic rats treated with insulin (115).

Early studies that focused on nNOS immunoreactivity in MD cells reported less intense immunohistochemical staining for constitutive NOS (presumably nNOS) in MD and glomerular arterioles in rats with diabetes for various durations (157). In concert with those findings, Keynan et al. (60) reported decreased NOS mRNA and diaphorase MD staining in rats at 7 days of diabetes. Furthermore, they found no differences in cortical eNOS between control and diabetic rats. Unlike most of the studies dealing with this issue, they found decreased urinary nitrite/nitrate excretion (U$_{\text{NOx,V}}$) in diabetic rats compared with controls.

Most recent analyses also do not provide unequivocal evidence. Ishii et al. (54) found no differences in cortical expression of NOS isoforms between diabetic animals and controls. Diaphorase staining also did not differ among groups. Another study (92), using whole kidney samples, found no change in nNOS and increased eNOS in female rats after 4 wk of diabetes. In addition, whole kidney eNOS and iNOS mRNAs evaluated by RT-PCR were unchanged in diabetic rats (112). Finally, increased eNOS expression was found in purified renal vascular trees of diabetic rats (26).

Thus it appears that consensus can be reached with respect to eNOS expression. Most of the available evidence suggests increased expression of this isoform in the diabetic renal cortex. There are disparate findings concerning nNOS, suggesting increased, normal, or decreased expression (Table 2). It should be noted that a decrease in nNOS in the diabetic renal cortex has been observed only in rats without insulin treatment. With the exception of one study (19), iNOS cortical

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**Table 2. Studies on effects of diabetes on NOS renal expression in vivo**

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Year</th>
<th>Insulin Treatment</th>
<th>Renal Compartment</th>
<th>Expression</th>
<th>DM Duration, wk</th>
<th>BG, mM</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yagihashi et al.</td>
<td>1996</td>
<td>–</td>
<td>MD</td>
<td>↓</td>
<td>6–32</td>
<td>~25</td>
<td>IH, NADPH</td>
</tr>
<tr>
<td>Choi et al.</td>
<td>1997</td>
<td>–</td>
<td>Cortex</td>
<td>↑</td>
<td>0.5–4</td>
<td>19–27</td>
<td>mRNA, protein</td>
</tr>
<tr>
<td>Souls et al.</td>
<td>1997</td>
<td>+</td>
<td>Cortex</td>
<td>↑</td>
<td>32</td>
<td>25–30</td>
<td>IH</td>
</tr>
<tr>
<td>Sugimoto et al.</td>
<td>1998</td>
<td>–</td>
<td>Cortex</td>
<td>↓</td>
<td>1–4</td>
<td>14–16</td>
<td>IH, NADPH</td>
</tr>
<tr>
<td>Sugimoto et al.</td>
<td>1999</td>
<td>–</td>
<td>Cortex</td>
<td>↑</td>
<td>26–52</td>
<td>23–25</td>
<td>mRNA, IH, NADPH</td>
</tr>
<tr>
<td>Veelken et al.</td>
<td>2000</td>
<td>–</td>
<td>Cortex</td>
<td>↓</td>
<td>2</td>
<td>17</td>
<td>mRNA, IH</td>
</tr>
<tr>
<td>Keynan et al.</td>
<td>2000</td>
<td>–</td>
<td>Cortex</td>
<td>↑</td>
<td>≥7</td>
<td>18–22</td>
<td>mRNA, IH, protein</td>
</tr>
<tr>
<td>Shin et al.</td>
<td>2000</td>
<td>–</td>
<td>Medulla</td>
<td>↑</td>
<td>6</td>
<td>19–33</td>
<td>mRNA, IH</td>
</tr>
<tr>
<td>Komers et al.</td>
<td>2000</td>
<td>+</td>
<td>MD</td>
<td>↑</td>
<td>4</td>
<td>16</td>
<td>IH, NOS-positive cell counts</td>
</tr>
<tr>
<td>Schwartz et al.</td>
<td>2001</td>
<td>+</td>
<td>Whole kidney</td>
<td>↔</td>
<td>4</td>
<td>19</td>
<td>mRNA, IH</td>
</tr>
<tr>
<td>de Vriese et al.</td>
<td>2001</td>
<td>+</td>
<td>Purified renal arteries</td>
<td>↔</td>
<td>6</td>
<td>26</td>
<td>Protein, NADPH, IH</td>
</tr>
<tr>
<td>Ishii et al.</td>
<td>2001</td>
<td>+</td>
<td>Cortex</td>
<td>↔</td>
<td>2</td>
<td>18</td>
<td>Protein, NADPH, IH</td>
</tr>
<tr>
<td>Onozato et al.</td>
<td>2002</td>
<td>–</td>
<td>Whole kidney</td>
<td>↔</td>
<td>4</td>
<td>22–25</td>
<td>IH, protein</td>
</tr>
</tbody>
</table>

nNOS, iNOS, and eNOS, neuronal, inducible, and endothelial NOS, respectively; MD, macula densa; IH, immunohistochemistry; NADPHD, NADPH diaphorase staining; ↔, no change compared with nondiabetic animals.
protein and mRNA expression have been found to be unchanged (54, 112) or barely detectable (119, 141) during the hyperfiltering stage. Two observations suggest that iNOS is detectable in the renal cortex of rats with long-term diabetes. iNOS was immunohistochemically detected in glomeruli of rats after 1 yr of diabetes but not in control age-matched animals (119). Another long-term study (32 wk) demonstrated weak glomerular expression in both control and diabetic rats (116) but no differences between the groups.

Disparate findings have also been reported in studies that determined renal NOS activity by a citrulline generation assay. Keynan et al. (60) found decreased cortical NOS activity. However, one might question the validity of the assay in this particular study because NOS activity was not detectable in the medulla, which should have much higher NOS activity than the cortex (154). In contrast, Omer et al. (91) and Ishii et al. (54) found increased cortical NOS activity in diabetic rats. Importantly, these studies differed in the absence (60) or presence (54, 91) of insulin treatment.

ROLE OF NO IN RENAL HEMODYNAMIC ALTERATIONS IN DIABETES

Early stages of diabetic nephropathy are associated with increases in GFR and variable increases in renal plasma flow (RPF) and filtration fraction, both clinically and experimentally (5). This “diabetic hyperfiltration” has been implicated in the pathogenesis of diabetic nephropathy both in humans as well as in animal models of diabetes (49, 83, 105, 160, 161). At the single-nephron level, diabetic hyperfiltration is characterized by disproportionately decreased afferent arteriolar resistance, resulting in elevated glomerular capillary pressure ($P_{GC}$) (50, 90, 160). Considering the renal hemodynamic actions of NO, this substance is a good candidate for mediating diabetic hyperfiltration. In this section, we will discuss studies exploring acute hemodynamic effects of modulation of NO synthesis in vivo (Table 3).

Most of the evidence concerning the role of NO in control of renal hemodynamics in diabetes was obtained using nonspecific NOS inhibitors. More recently, several studies have attempted to test the activity of individual NOS isoforms with newly available isoform-specific inhibitors. In a number of these studies, GFR and RPF were measured as clearances of inulin and PAH using constant-infusion techniques. Despite the explosive developments in molecular biology, we consider such evidence as the gold standard when evaluating the role of a particular substance in the control of renal hemodynamics in vivo. In addition

Table 3. Studies on effects of NOS modulation on renal hemodynamics in models of diabetes

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Year</th>
<th>Insulin Treatment</th>
<th>Intervention</th>
<th>Renal Response in DM</th>
<th>Assessment of Renal Function</th>
<th>DM Duration, wk</th>
<th>BG, mM</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>King et al.</td>
<td>1990</td>
<td>–</td>
<td>1-NMMA</td>
<td></td>
<td>Inulin/PAH</td>
<td>2–3</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td>Kiff et al.</td>
<td>1991</td>
<td>–</td>
<td>Ach,BK,GTN</td>
<td></td>
<td>Doppler</td>
<td>2</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td>Kiff et al.</td>
<td>1991</td>
<td>–</td>
<td>1-NNAME</td>
<td></td>
<td>Doppler</td>
<td>2</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td>Wang et al.</td>
<td>1993</td>
<td>–</td>
<td>Ach</td>
<td></td>
<td>Doppler/Inulin</td>
<td>2–3</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Bank and Aynedjian</td>
<td>1993</td>
<td>–</td>
<td>1-NLA</td>
<td></td>
<td>Inulin/PAH</td>
<td>1.5–2</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Tolins et al.</td>
<td>1993</td>
<td>+</td>
<td>1-NNAME</td>
<td></td>
<td>Inulin/PAH</td>
<td>6</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Komers et al.</td>
<td>1994</td>
<td>+</td>
<td>1-NNAME</td>
<td></td>
<td>Inulin/PAH</td>
<td>3–4</td>
<td>25–30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GTN</td>
<td></td>
<td></td>
<td>Inulin/PAH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mattar et al.</td>
<td>1996</td>
<td>+</td>
<td>1-NNAME</td>
<td></td>
<td>Inulin/PAH</td>
<td>4</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Goor et al.</td>
<td>1996</td>
<td>–</td>
<td>1-NNAME</td>
<td></td>
<td>Ccr</td>
<td>2</td>
<td>17</td>
<td>Intact kidney</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ccr</td>
<td></td>
<td></td>
<td>Ischemic kidney</td>
</tr>
<tr>
<td>Sugimoto et al.</td>
<td>1998</td>
<td>–</td>
<td>1-NNAME</td>
<td></td>
<td>Ccr</td>
<td>1–4</td>
<td>14–16</td>
<td></td>
</tr>
<tr>
<td>Omer et al.</td>
<td>1999</td>
<td>+</td>
<td>1-NLA</td>
<td></td>
<td>Inulin/PAH</td>
<td>2</td>
<td>35</td>
<td>Virgin/pregnant females</td>
</tr>
<tr>
<td>Pflueger et al.</td>
<td>1999</td>
<td>–</td>
<td>1-NAMA</td>
<td></td>
<td>Videomicroscopy</td>
<td>1–2</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Pflueger et al.</td>
<td>1999</td>
<td>–</td>
<td>1-NAMA</td>
<td></td>
<td>Videomicroscopy</td>
<td>4–5</td>
<td>20</td>
<td>Adenosine</td>
</tr>
<tr>
<td>Komers et al.</td>
<td>2000</td>
<td>+</td>
<td>SMTC</td>
<td></td>
<td>Inulin/PAH</td>
<td>4</td>
<td>16–17</td>
<td>Systemic inhibition</td>
</tr>
<tr>
<td>Veenen et al.</td>
<td>2000</td>
<td>–</td>
<td>1-NNAME</td>
<td></td>
<td>Ccr</td>
<td>2</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Komers et al.</td>
<td>2000</td>
<td>+</td>
<td>SMTC</td>
<td></td>
<td>Inulin/PAH</td>
<td>4</td>
<td>17</td>
<td>Local inhibition</td>
</tr>
<tr>
<td>de Vries et al.</td>
<td>2001</td>
<td>+</td>
<td>1-NNAME</td>
<td></td>
<td>Videomicroscopy</td>
<td>6</td>
<td>26</td>
<td>Hydronephrotic kidney</td>
</tr>
<tr>
<td>Ito et al.</td>
<td>2001</td>
<td>–</td>
<td>1-NNAME</td>
<td></td>
<td>Inulin/PAH</td>
<td>2</td>
<td>17–24</td>
<td></td>
</tr>
<tr>
<td>Schwartz et al.</td>
<td>2001</td>
<td>+</td>
<td>1-NNAME</td>
<td></td>
<td>Inulin/PAH</td>
<td>4</td>
<td>19</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1-NIL</td>
<td></td>
<td>Inulin/PAH</td>
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</table>

N$^4$-monomethyl-l-arginine (1-NMMA), l-NNAME, and N$^6$-nitro-l-arginine (1-NLA): nonspecific NOS inhibitors; S-methyl-l-thiocitrulline (SMTC) and 7-nitro indazole (7-N): nNOS-specific inhibitors; 1-N$^6$-(1-iminoethyl)lysine (1-NIL), specific iNOS inhibitor; BK, bradykinin; GTN, glyceryl trinitrate; Ccr, creatinine clearance.

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to measurements of renal hemodynamics, these studies have also often relied on measurements of UNOxV and urinary cGMP as markers of renal NO production.

To our knowledge, the first in vivo evidence indicating that modulation of NO synthesis could influence renal hemodynamics in diabetes was reported in an abstract by King et al. (62). Their preliminary data showed a major renal vasoconstrictor effect of a pressor dose of NOS inhibitor, suggesting greater NO dependence of renal hemodynamics in diabetic compared with control rats. Full papers by other groups addressing this issue followed shortly. Bank and Aynedjian (8) found an increase in UNOxV in hyperfiltering diabetic rats, consistent with increased NO production. However, when administered increasing doses of NLA, both diabetic and normal rats had similar decreases in GFR and RPF. Despite these disparate findings, the authors concluded that NO synthesis was increased in hyperfiltering diabetic rats and that excessive NO synthesis contributed to hyperfiltration. Tolins et al. (128) also found increased UNOxV in hyperfiltering diabetic rats. However, NOS inhibition with L-NAME normalized GFR in diabetic rats and induced greater vasoconstrictor responses under various perfusion pressures. Importantly, perfusion pressure was modified independently of the systemic blood pressure changes that would usually be associated with systemic NOS inhibition. Thus both excretion of stable metabolites of NO and renal hemodynamic responses suggested enhanced renal generation of NO and its contribution to the pathogenesis of diabetic hyperfiltration.

We evaluated the effects of L-NAME in conscious control and hyperfiltering diabetic rats (63). At a low dose of L-NAME, diabetic rats demonstrated a blunted mean arterial pressure (MAP) response and a proportionately significant reduction in GFR and RPF compared with controls. At a supramaximal dose, the L-NAME-induced rise in MAP was similar in diabetic and control rats. However, renal vasoconstriction was greater in the diabetic animals. UNOxV was increased in diabetic rats and significantly reduced by low-dose L-NAME. Furthermore, diabetic rats demonstrated no response to the NO donor glyceryl trinitrate (GTN), which induced significant renal vasodilation in control rats. We interpreted the lack of a GTN effect in the diabetic kidney as a further indication of enhanced NO production and/or signaling that cannot be significantly altered by additional NO. Interestingly, this phenomenon was later quoted in support of impaired NO actions in the diabetic kidney (96). These results, in accordance with the previous study, suggested a role for NO in the pathogenesis of hyperfiltration and increased renal NO generation in diabetes. Similar data, i.e., significant reduction in hyperfiltration in anesthetized diabetic rats in response to L-NAME, were then reported by Mattar et al. (78) and in several studies that focused on other issues (118, 141). Furthermore, extensive studies by Omer et al. (91) confirmed our findings in virgin and pregnant normal and diabetic rats. Both groups demonstrated marked hyperfiltration that was nearly normalized by L-NNA. Moreover, substantial increases in RPF in diabetic animals, further enhanced by pregnancy, also showed marked sensitivity to NOS inhibition. Similar to our studies (63), the MAP response was attenuated in virgin diabetic animals compared with controls. These hemodynamic observations were associated with parallel changes in UNOxV and increased baseline NO renal generation as assessed by the citrulline generation assay. Interestingly, this group also found an enhanced response to ACh in aortic rings from diabetic rats (91).

Most recently, the effects of nonspecific NOS inhibition were evaluated in hydronephrotic kidneys of control and moderately hyperglycemic female rats at 6 wk of diabetes (26). This model was used to allow intravital microscopy for evaluations of changes in renal vascular diameters. Increased vasoconstrictor responses to L-NAME in diabetic rats, suggesting local hyperproduction and activity of NO, were apparent in all segments of the renal vasculature. One might argue that using the hydronephrotic kidney model involves factors other than the diabetic milieu that would alter local NO production and therefore complicate interpretation of the results. However, renal vascular responses were in good accord with eNOS protein expression determined in vascular trees of intact kidneys.

Not all authors have found enhanced whole kidney hemodynamic responses to NO inhibition. Using Doppler probes, Kiff et al. (61) found similar responses to L-NAME in conscious control and diabetic rats. Another study (37) reported rather controversial observations. In basal conditions, diabetic rats demonstrated marked increases in creatinine clearance (CCr) and UNOxV compared with controls. L-Arg increased UNOxV in controls but not in diabetic rats. L-NAME had no effect in controls but decreased UNOxV in diabetic animals. In further studies, uninephrectomized control and diabetic rats were evaluated after 60 min of renal ischemia. Control rats responded with less severe decreases in CCr and higher UNOxV, whereas diabetic animals demonstrated an opposite trend that was further aggravated by L-NAME. Despite the finding in basal conditions, the authors concluded that renal NO production is defective in diabetes (37). Given the pathophysiological complexity of ischemic renal failure, it is also unclear whether kidneys after 60 min of total occlusion of the renal artery represent an appropriate model for studies of the NO system in diabetes.

Pfueger et al. (96) applied videomicroscopy in control and STZ-diabetic rats to assess cortical and medullary blood flow in superficial cortical capillaries and papillary vasa recta. Diabetic rats demonstrated basal increases in these parameters, as well as increases in GFR (measured as inulin clearance in the contralateral kidney) and plasma NOx levels. UNOxV did not differ between control and diabetic rats. Despite increased blood flow and a higher GFR, diabetic rats showed attenuated responses of the cortical and papillary microcirculations to both systemic NOS inhibition with L-NMMA and stimulation of NO synthesis with L-Arg. The unresponsiveness of NO synthesis inhibition was greatest in cortical capillaries. Thus, despite baseline
hyperperfusion, these data suggest a defect in renal, and particularly renal cortical, NO production or action in diabetes in vivo. In a parallel study, the same group reported increased sensitivity of diabetic renal vasculature to adenosine due to defective counterregulatory NO production (97). Of note, increases in GFR observed in contralateral kidneys in diabetic rats were normalized by NOS inhibition. It is not clear how measurements of a single capillary diameter and red blood cell velocity relate to the traditional methods for determining renal function, because the changes in GFR were not reflected by the effects of NOS inhibition on cortical capillaries in the contralateral kidney.

Whereas the preceding studies relied on nonspecific NOS inhibitors, the contribution of individual NOS isoforms to NO production in the diabetic kidney has only recently been evaluated. Research in this direction has been accelerated by the availability of new inhibitors that selectively block NOS isoforms. Studies by Wang et al. (146) represent a less frequently used approach to investigate in vivo whole kidney responses to NO modulation. In these studies, the authors evaluated responses to intravenous infusion of ACh. Therefore, these studies could be interpreted as exploring isoform-specific NO production (eNOS). The renal vasodilator response to ACh was diminished in diabetic rats but not in normoglycemic diabetic rats. Acute treatment with insulin did not restore the response to ACh, although the blood glucose level was normalized. These in vivo experiments further supported the in vitro data by this group (146), suggesting reduced NO bioavailability and/or impaired signaling in the renal vascular endothelium and the previously discussed possible defects in receptor-mediated eNOS stimulation.

There is abundant literature suggesting defective endothelium-dependent (i.e., eNOS dependent) NO production or function in diabetes, in apparent contrast to observations of substantial NO dependency of renal hemodynamics in hyperfiltering rats, as revealed with nonspecific NOS inhibitors. We hypothesized that increased renal nNOS activity could explain this paradox. This isoform is expressed in MD cells (7, 150), and nNOS-derived NO in MD decreases predominantly afferent arteriolar tone (51, 150), contributes to control of intraglomerular pressure (124, 150), and counteracts afferent vasoconstriction induced by activation of tubuloglomerular feedback (TGF) (51). We explored the acute effects of systemic nNOS inhibition with the specific nNOS inhibitor S-methyl-L-thiocitrulline (SMTC) (89) in diabetic rats (66). SMTC induced stronger renal vasoconstriction responses in diabetic compared with control animals. Renal vasoconstriction was partly opposed by ANG II AT1 receptor blockade, suggesting an interaction of NOS-derived NO with ANG II. Further studies were designed to diminish the possible systemic effects of SMTC (64). When administered directly into the abdominal aorta above the left renal artery, SMTC did not influence MAP but nearly normalized GFR in hyperfiltering diabetic rats. These observations indicate that NOS contributes to altered renal NO production and hemodynamics in experimental diabetes. Renal hemodynamic effects of SMTC were attenuated in normoglycemic diabetic rats, suggesting that alterations in nNOS activity are related to the level of metabolic control. Moreover, the nonspecific NOS inhibitor L-NAME did not influence GFR but further decreased RPF in diabetic rats pretreated with SMTC. These data suggest that nNOS is the major isoform responsible for hyperfiltration, although other isoforms act in concert with nNOS in the control of renal perfusion.

Similar results regarding the role of nNOS were more recently reported by Ito et al. (55), using a different NOS inhibitor [7-nitro indazole (7-NI)]. Furthermore, data by Schwartz et al. (112), showing enhanced renal hemodynamic response to a nonspecific NOS inhibitor, in conjunction with an absence of response to the iNOS blocker L-N6-(1-iminoethyl)lysine (L-NIL) and unchanged renal iNOS and eNOS mRNA expressions, indirectly point to nNOS. Further indirect support for the role of NOS in diabetic alterations in glomerular hemodynamics can be derived from observations that the activity of TGF is blunted in diabetes (135). The physiological roles of NOS-derived NO (51) and its suggested higher production by MD cells in diabetes are consistent with this abnormality. In addition, Veelken et al. (141) reported the lack of effect of a selective iNOS inhibitor, L-NIL, in contrast to the effect of L-NAME, on renal hemodynamics in conscious STZ-diabetic rats. Because cortical eNOS expression was increased in diabetic rats, the authors suggested the role of eNOS in the pathogenesis of hyperfiltration. However, these findings do not exclude the possibility that nNOS is involved in the process, because the reduction of GFR was achieved with a nonspecific NOS inhibitor.

Unlike in vitro and in vivo studies focusing on renal hemodynamic expression, we are able to identify some differences in in vivo hemodynamic studies, suggesting enhanced renal NO production/activity in diabetes and those studies reporting opposite data. There is a clear distinction between the two lines of evidence with respect to methods of measurements of renal function. In those studies that used formal clearance techniques to determine GFR and RPF, diabetic rats demonstrated hyperfiltration and enhanced responses to NOS inhibition, suggesting increased renal production and/or activity. In contrast, studies with contrary evidence used either Ccr or techniques requiring extensive instrumentation (96, 97).

Another factor that could influence the outcome of these studies is the presence or absence of insulin treatment. Those investigators who used insulin treatment to achieve moderate hyperglycemia have generally found hyperfiltration and enhanced hemodynamic responses to NOS inhibition. One might argue that decreased renal hemodynamic responsiveness to NOS inhibitors has also been observed in moderately hyperglycemic rats. However, in those studies, despite an absence of exogenous insulin treatment, residual endogenous insulin secretion prevented severe hyperglyc-
cemia (96, 97). As discussed below, the combination of moderate hyperglycemia and exogenous insulin treatment may be a crucial factor creating the milieu for NO-dependent diabetic hyperfiltration. This interpretation is in accord with the available micropuncture data (110). An exception to this rule may be the elegant studies of Carmines et al. (90, 111). However, the technique used by that group (evaluation of arteriolar diameters in isolated blood-perfused nephrons) is still an in vitro technique. Consequently, arteriolar and glomeruli in diabetic animals are not exposed to the same metabolic milieu as occurs in vivo.

Many in vivo studies have buttressed their conclusions of enhanced renal NO production by demonstration of increased $U_{NOX}$. However, these measurements should be interpreted with caution. Considering present knowledge of the distribution of NOS activities in the kidney (154), and studies by Suto et al. (120) that directly addressed this issue, it is clear that measurements of urinary NOx can hardly reflect NO changes in the cortex. Although some attempts to directly determine renal NO production in vivo have been communicated, our present knowledge still relies mostly on indirect indicators of renal NOS activity. Direct measurements of NO in the kidney represent some of the challenges for future research.

Pieper (98) has suggested that the duration of diabetes is an important determinant for NO production in the vascular system, evolving from an increase during the early stages, followed by a period of normal production, and then to a decrease in production as the duration of the disease increases. However, this construct does not really explain the discrepancies in published data regarding either renal NOS expression or in vivo studies of hemodynamics. The evidence remains controversial irrespective of the duration of diabetes.

**Effects of Long-Term Modulation of NO Synthesis in the Diabetic Kidney**

One would expect that long-term modulation of NO would ultimately answer many questions regarding the role of NO in the development of diabetic nephropathy. However, even these types of studies have not provided unequivocal data. Several studies have suggested a nephroprotective role of NO in the development of nephropathy in diabetic rats. Reyes et al. (103) found lower proteinuria and amelioration of hyperfiltration in STZ-diabetic rats after 14 wk of L-Arg treatment. However, this study reported several findings that are difficult to interpret. Administration of L-Arg did not lead to increased cGMP and $U_{NOX}$; in fact, these parameters were lower in treated rats. Thus the study failed to provide proof that the decrease in proteinuria was mediated via increased renal NO production. Paradoxically, in both vehicle- and L-Arg-treated diabetic rats, urinary protein excretion was higher at baseline than after treatment, and the achieved values exceeded levels seen in animals with severe glomerulosclerosis. Furthermore, histological evaluation did not reveal any differences in glomerulosclerosis scores between L-Arg-treated and vehicle-treated rats.

Further evidence for a possible renoprotective role of NO in diabetes was reported by Craven et al. (21). STZ-diabetic rats received nonpressor doses of L-NAME for 4 wk. At the end of the study, L-NAME-treated rats had slightly but significantly higher albuminuria, although still in the microgram range. Importantly, renal transforming growth factor-$\beta$ (TGF-$\beta$) was increased in treated rats. However, the study was too short to determine whether chronic nonpressor L-NAME could accelerate the course of nephropathy. This issue was addressed by Soulis et al. (116), who found no effect of chronic (6 mo) treatment with a nonpressor dose of L-NAME on the progression of nephropathy in STZ-diabetic rats. In addition, corresponding to our acute studies (64), our preliminary data suggest that long-term nNOS inhibition with a nonpressor dose of SMTC modestly retards development of renal injury in diabetes (67).

**POSTTRANSLATIONAL MODULATION OF NOS ACTIVITY: IMPACT OF DIABETES**

In the previous sections, we focused on individual studies, pointed out some differences, and attempted to find some explanation for disparate findings in studies with generally similar designs. In the following paragraphs, we will discuss additional controversial issues that arise when one analyzes particular aspects of renal NO physiology and pathophysiology with respect to the pathogenesis of diabetic nephropathy. To shed light on the issues, we will include some evidence from the extrarenal literature. Although we will discuss the following issues separately, it is apparent that they represent a conglomerate of interacting mechanisms.

**Changes in eNOS Conformation and Generation of ROS**

To function as NO-producing enzymes, NOS requires a battery of cofactors, conformational changes, and fatty acid acylations responsible for membrane targeting of the enzyme (reviewed in Refs. 39 and 81). A traditional pathway of eNOS activation involves receptor stimulation by agonists such as ACh or bradykinin, resulting in a mobilization of intracellular Ca$^{2+}$ and consequent interaction of calmodulin and eNOS, releasing the enzyme from an inhibitory complex with caveolin-1 (58). On more prolonged agonist stimulation, eNOS may be released from its membrane location and translocated to other subcellular compartments. This reversible translocation may represent a mechanism to downregulate or disconnect the enzyme from receptor occupancy (76).

In addition to synthesizing NO, purified NOS catalyzes $O_2$ formation under certain conditions, such as tetrahydrobiopterin (BH4) or L-Arg deficiency (102, 138, 139, 155, 156). In this "uncoupled state," electrons flowing from the NOS reductase domain to the oxygenase domain are diverted to molecular oxygen rather than to L-Arg (139, 148, 155). Thus NOS acts as an...
enzyme with dual functions, capable of producing both NO and ROS. It has been proposed that formation of a dimer is crucial for NO production by NOS (45). BH4 and calmodulin have been suggested as molecules essential for dimer formation, although there the evidence for BH4 is controversial (45).

Enhanced production of ROS appears to be an important mechanism in the pathophysiology of diabetic complications, including nephropathy. Their role has been validated in long-term studies (71, 80). ROS may be involved in diabetes-induced alterations in lipids and proteins, cellular signaling (40), as well as inactivation of NO. After generation by these enzymes, O₂⁻ may undergo reaction with NO, resulting in formation of cytotoxic ONOO⁻, or may be transformed into the more stable radical H₂O₂. This reaction is catalyzed by SOD. H₂O₂ is further metabolized by catalase. Two studies determined nitrosylated protein (NT) expression in diabetic kidneys by Western blotting as a measure of ONOO⁻ formation (54, 92). Both studies found increases in this parameter in diabetic kidneys, indicating enhanced interaction between NO and O₂⁻. However, those studies did not provide information about localization of NT in the kidney. Furthermore, in the latter study (93), renal tissue was analyzed as a whole without separation of cortex and medulla. Considering high NO activities in the medulla, it is not clear how much ONOO⁻ was formed in that compartment. Post-mortem human data show increased NT formation in proximal tubules of patients with long-standing diabetes compared with kidneys from patients with glomerulonephritis or normal kidneys (125). Thus far, then, there is no proof of enhanced NT formation in the renal vascular tree in diabetes.

There are several sources of ROS (108). Some investigators have addressed the question of whether eNOS could be an important source of ROS in diabetes. Co-sentino et al. (20) reported increased eNOS expression associated with parallel increases in NO and O₂⁻ in human aortic endothelial cells exposed to high glucose (22.2 mM) for 5 days. Hink et al. (46) found that impaired endothelium-dependent vasodilation in STZ-diabetic rats was associated with increased vascular eNOS expression and ROS production. Because the increase in ROS production by diabetic vessels was corrected by NOS inhibition, the results suggested uncoupled eNOS as an important source of ROS in diabetic arteries. In addition, the authors showed that PKC inhibition might prevent eNOS uncoupling. The mechanisms described in aortic tissue could also be active in the kidney. Indeed, Ishii et al. (54) have recently demonstrated increased NOS activity and larger quantities of O₂⁻ produced in the diabetic rat kidney cortex. Further evidence suggests that eNOS uncoupling could be prevented by BH4, identifying diabetes as a state of BH4 depletion (99).

In an aforementioned in vitro report, L-Arg had improved basal, but not ACh-stimulated, cGMP production by isolated glomeruli (23). This phenomenon suggests a specific glucose-induced defect in receptor-mediated eNOS activation, mediated by factors already mentioned (23) or, for example, by ROS-induced alterations in eNOS-caveole interactions, eNOS cellular localization, and cofactor integrity (56, 95).

The fact that eNOS undergoes important posttranslational changes and protein-protein interactions documents the importance of more detailed experimental approaches in evaluating NOS expression in the diabetic kidney. For example, the changes in renal expression of NOS mRNA or total protein in the diabetic kidney may not reflect the functional status of the enzyme. Because most of the evidence concerning eNOS expression in the diabetic kidney has not taken into account the quaternary structure of NOS and other posttranslational modifications, it is difficult to draw links between most of the available knowledge concerning renal NOS expression and its function. Considering the disparate findings in some studies focusing on eNOS-mediated vascular reactivity in diabetes, it is possible that the results depend on the actual balance between NO and O₂⁻. This balance could be influenced by a number of factors, including the experimental technique.

We have recently attempted to address some of these issues. Our preliminary data suggest alterations in some of these posttranslational modifications and other functionally important characteristics, such as decreased formation of the NO-producing eNOS dimer, alterations in membrane eNOS targeting, and eNOS-caveolin-1 interaction in the diabetic renal cortex (65). Further documenting the need for more detailed analytic approaches to assess renal NOS, a preliminary report by Carmines et al. (15) demonstrated reduced renal cortical expression and function of heat shock protein 90 in diabetes, another protein cofactor facilitating eNOS catalytic activities.

Considering the data from long-term studies (71, 80), one would not question the contribution of ROS to the development of nephropathy, although beneficial effects of antioxidant treatment remain to be established in large clinical trials. However, the data suggesting the contribution of NO-ROS interactions to alterations in renal vasmotor function in diabetes raise several questions. First, this evidence suggests that O₂⁻, a renal vasoconstrictor, decreases bioavailability of NO or limits the buffering capacity of NO against vasoconstrictors (111). Therefore, one would expect that the net effect of this imbalance would result in renal vasoconstriction. However, this is difficult to reconcile with the fact of diabetic hyperfiltration. Unlike most of the other groups, Carmines et al. (16) have offered an explanation for this contradiction. They suggested that a decrease in afferent arteriolar tone in diabetes is a result of a functional defect in afferent arteriolar voltage-gated Ca²⁺ channels, leading to impaired vasoconstriction. In addition, studies in isolated nephrons and arterioles (90, 109) that provided the most compelling evidence for the ROS-NO interaction in the control of renal hemodynamics in diabetes did not take into account NO from nonendothelial sources, such as MD.
Second, we are not aware of any evidence demonstrating the effect of $O_2^-$ scavenging with SOD or a SOD mimetic on basal arteriolar tone in diabetic rats. Considering the NO-$O_2^-$ interaction, one would expect enhanced SOD-induced afferent vasodilator responses not only during ACh stimulation (109) but also in the basal, unstimulated state. However, the SOD mimetic tempol did not alter basal afferent tone in diabetic rabbits (109). Corresponding to vascular reactivity studies, antioxidant treatment did not alter basal mesangial cell NO production in high-glucose media (132).

Third, $O_2^-$ dismutation by SOD results in formation of $H_2O_2$. Unlike $O_2^-$, $H_2O_2$ acts as a renal vasodilator (108, 137). Provided that renal arteriolar NO and $O_2^-$ are produced in large quantities, vascular responses to SOD are determined by protection of NO from quenching, by the elimination of the vasoconstrictor actions of $O_2^-$, and by further vasodilator effects of $H_2O_2$. It has been suggested that $H_2O_2$ could be one of putative endothelial vasodilators often referred to as endothelium-derived hyperpolarizing factor (137). Therefore, the effects of SOD on ACh-stimulated vascular reactivity may be attributable, in part, to $H_2O_2$. However, it should be noted that the role of $H_2O_2$ is not supported by data obtained with tempol. This substance has similar effects to those of SOD on NO-dependent responses in diabetes, but it also enhances $H_2O_2$ dismutation (72), presumably eliminating the contribution of $H_2O_2$.

Fourth, the most persuasive evidence for potentiation of NO-dependent vasodilation by $O_2^-$ quenching was obtained in vitro. In vitro systems may be predisposed to the formation of ROS. One of those mechanisms could be a lack of L-Arg in cultivation media or perfusion solutions. As already mentioned, eNOS uncoupling could be enhanced in situations with reduced substrate availability (139). In contrast, in vivo studies demonstrated that antioxidant treatment may normalize hyperfiltration in diabetes (71). Considering the decrease in the filtration fraction in diabetic rats treated with antioxidants observed in that study, one would expect efferent arteriolar effects of antioxidant treatment. These data suggest that these effects in the renal microvasculature are attributable not only to protection of NO from quenching but also to other mechanisms. One of the mechanisms compatible with the long-term glomerular hemodynamic action of antioxidants could be, for example, inhibition of ANG II signaling via ROS (40).

**Effects of Diabetes on Signaling Pathways That Modulate Renal Activity and/or Expression of NOS**

In vitro evidence has demonstrated that eNOS is regulated by coordinated signaling via phosphorylation and dephosphorylation of tyrosine, serine, and threonine amino acid residues (32). For example, phosphorylation of Ser1177/1179 leads to activation of the enzyme (42), whereas phosphorylation of Thr495 has an opposite effect (33). Diabetes, by altering various signaling pathways, may theoretically influence the phosphorylation status of eNOS and presumably also of other NOS isoforms.

The impact of PKC activation in the pathophysiology of nephropathy has been well established (70). Phosphorylation of eNOS by PKC inhibits its catalytic activity (47), and this inhibition is accomplished by phosphorylation of Thr495 and dephosphorylation of Ser1177 (82). Specifically, the PKC-β isoform has been implicated in the pathophysiology of complications (69). This isoform inactivates eNOS in endothelial cells and microvessels of insulin-resistant Zucker rats (75). Identification of the link between PKC and eNOS activity provides strong support for the concept of NO insufficiency in the diabetic kidney (Fig. 1). More recently, other signaling pathways have been identified as modulators of NOS activity along with evidence suggesting alterations of these pathways in diabetes.

We have repeatedly emphasized the presence or absence of insulin treatment preventing severe hyperglycemia as a possible contributor to disparate findings in a number of the preceding studies. In our opinion, the crucial role of exogenous insulin is not merely in modulating blood glucose levels but also in its ability to influence NO synthesis. Indeed, early after induction of diabetes, residual secretion of insulin may be sufficient to achieve moderate hyperglycemia. However, to achieve given levels of blood glucose, the amount of exogenous insulin could be substantially greater compared with the amount of endogenously secreted hormone. Thus, acting via Akt, insulin could be an independent activator of eNOS in various tissues including the kidney. This pathway would not be reflected, for example, in studies using stimulation with ACh, a widely accepted approach for testing eNOS function. We cannot exclude that impaired agonist-induced NO production, as observed in a number of studies, could be counterbalanced by alternative signaling pathways, such as Akt. Local activation of Akt by hyperinsulinemia may be an example of such a situation (Fig. 1).

Some investigators have suggested a defect in Akt signaling as one of the sites of insulin resistance (18, 73), a hallmark feature of type 2 diabetes. An important issue is whether resistance to metabolic actions of insulin affects not only metabolic but also vasoactive or renal actions of the hormone. An increase in Akt expression in the kidney in insulin-resistant mice suggests that these two processes may be separated (31). However, opposite evidence suggesting resistance to insulin-induced NOS activation also exists (75).
thermore, another line of in vitro evidence suggests that in high glucose, the Akt-dependent phosphorylation site responsible for eNOS activation can be modified by N-acetyl glucosamine (30), in a process linked to mitochondrial O$_2$ production. There is also conflicting evidence with respect to the possible impact of PKC on Akt signaling, resulting in eNOS activation (75, 133).

Unlike eNOS, there is much less evidence on the effects of insulin and diabetes-induced signaling pathways on nNOS activity. However, there is indirect evidence from other tissues suggesting that insulin could activate nNOS. For example, pial arteriolar vasodilation associated with insulin-induced hypoglycemia is mediated by nNOS-derived NO (106). Similar to eNOS, rat nNOS contains an Akt-dependent phosphorylation motif (34). However, phosphorylation of this motif has not been shown to significantly modulate NO production by the enzyme (34).

Although cellular signaling pathways possibly modulating nNOS activity in the diabetic kidney have not been elucidated, other mechanisms involved in renal nNOS regulation have been identified. Importantly, some of these mechanisms may be active in the diabetic kidney. Under physiological conditions, sodium delivery to the distal tubule is the major acute determinant of nNOS activity in the MD cells (51, 149). However, based on micropuncture studies by Vallon et al. (135) showing a decrease in solute content in early distal tubular fluid in diabetic rats, stimulation of nNOS by increased solute delivery to the MD in diabetes is unlikely. Welch and Wilcox (147) reported that blunting of TGF responses by NO could be limited by l-Arg availability in the tubular lumen and by its uptake via the y$^+$ transport system. To our knowledge, the availability of l-Arg in diabetic compared with normal kidneys remains unknown. Indirect clues that could help elucidate this issue are rather conflicting. Plasma l-Arg levels are decreased in diabetes (100), but urinary l-Arg excretion has been reported to be markedly increased (103). Renal nNOS is chronically activated in parallel with the renin-angiotensin system (RAS) in such pathophysiological states as two-kidney, one-clip hypertension, furosemide treatment, or dietary sodium restriction (12). Assuming that a low-sodium diet decreases distal sodium delivery, this mechanism corresponds to the situation in the distal tubule as described by Vallon et al. (135) in diabetic rats and thus represents a possible pathway resulting in activation of nNOS.

**NO AND RENAL STRUCTURAL CHANGES IN DIABETES**

In the diabetic kidney, most cells undergo hypertrophy. This process, together with accumulation of extracellular matrix, underlies renal structural changes in diabetes that are characterized by mesangial expansion (93), later development of glomerulosclerosis, and by tubular hypertrophy and later interstitial fibrosis (36). Data reported by several groups have provided evidence that this hypertrophy is, at least in part, attributable to altered cell cycle regulation. This complex process, reviewed elsewhere (151), is associated with increased expression of cyclin-dependent kinase (CDK) inhibitors, such as p21$^\text{Cip1}$ and p27$^\text{Kip1}$ (74, 153), resulting in G$_1$-phase arrest (151). These molecules can be induced by glucose and other mediators of the diabetic milieu, such as glucose-TGF-β or RAS-TGF-β pathways (151), molecules whose importance in the pathophysiology of diabetic nephropathy is well documented (2).

Antiproliferative actions of NO on mesangial cells or vascular smooth muscle cells, and its tendency to shift
cells into the hypertrophic phenotype, have been recognized for over a decade (35, 57, 144). Later studies have provided evidence for mechanisms responsible for these actions (41, 53, 114, 122). NO directly modulates these actions (41, 53, 114, 122). NO directly modulates p21Cip1 and inhibition of cyclin-dependent kinase 2 activity. Thus it appears that NO growth actions in the kidney display substantial similarities to the diabetic milieu. Importantly, these effects of NO are independent of cGMP generation (122). Furthermore, TGF-β can induce eNOS in vitro (52) as well as in vivo in both diabetic (94) and nondiabetic tissues (101, 158). TGF-β-induced activation of eNOS may involve Akt kinase signaling (17, 94) (Fig. 1).

Another factor implicated in the pathogenesis of diabetic nephropathy and signaling via Akt is VEGF (27). Reviewing the data on VEGF well illustrates the controversial nature of evidence discussed in this review. There is abundant evidence suggesting NO as a mediator of VEGF actions (44, 48, 136) (Fig. 1). However, some actions of VEGF presumably mediated by NO, such as endothelial proliferation (87), are not typically observed in the diabetic kidney and are not in accordance with the effects of NO on cellular trophic status discussed above. In contrast, other effects, such as local NO-dependent increases in vascular permeability, vasodilation, or glomerular hypertrophy, are well consistent with diabetic renal pathophysiology (27, 126). In any case, provided that the hypothesis about the role of VEGF is valid, it is hard to reconcile with those findings suggesting a NO deficit in the diabetic kidney.

As with other issues discussed in this review, convincing contradictory evidence has also been reported in this area, suggesting that a NO deficit may underlie the development of renal structural changes in diabetes. Several in vitro reports have indicated that NO can interfere with pathways, resulting in TGF-β induction by high glucose or other mechanisms active in the diabetic kidney (24, 117). It has recently been reported that TGF-β suppression by NO in mesangial cells cultured in high glucose is mediated by downregulation of thrombospondin-1 (145). This view was further supported by observations that the RAS-TGF-β axis is involved in mediating organ injury during chronic NOS inhibition in diabetic (21) and nondiabetic models (59, 129). Thus mechanisms discussed in this section suggest that both increased as well as decreased renal NO activity may result in characteristic changes in diabetic renal morphology.

**SUMMARY**

We have reviewed a wide spectrum of findings and issues that have amassed concerning the pathophysiology of the renal NO system in diabetes. It is apparent that it might be practically impossible to reconcile such complex and controversial evidence and find a unifying scenario characterizing these processes. However, we believe that some patterns are emerging. One of major general controversies exists between the in vitro findings, which generally suggest decreased bioavailability of NO in the diabetic kidney, and in vivo observations, which tend to suggest enhanced renal NO production and/or activity in diabetes, at least in the early stages. We believe that the diabetic milieu is complex and that in vitro approaches may miss some important mechanisms that modulate NO activity in a particular system. On the other hand, these studies are indispensable in the identification of precise mechanisms resulting in alterations of the NO system in diabetes. Most importantly, in vitro studies describe processes whereby high glucose levels, a hallmark metabolic feature of diabetes, exert deleterious effects on NO bioavailability.

There is little doubt that severe diabetes with profound insulinopenia can be viewed as a state of general NO deficiency, including in the kidney. However, it is important to note that we focus our hypotheses and conclusions on the events occurring during moderate glycemic control, with some degree of treatment with exogenous insulin (Fig. 1). This situation represents more the clinically applicable model most closely resembling the situation in patients destined for development of nephropathy.

Diabetes triggers mechanisms that in parallel enhance and suppress NO bioavailability in the kidney (Fig. 1). We hypothesize that during the early phases of nephropathy, the balance between these two opposing forces is shifted toward NO. This plays a role in the development of characteristic hemodynamic changes and may contribute to consequent structural alterations in glomeruli. Both eNOS and nNOS can contribute to altered NO production. These enzymes, in particular eNOS, can be activated by Ca²⁺-independent and alternative routes of activation that may be elusive in traditional methods of investigation. As the duration of exposure to the diabetic milieu increases, factors that suppress NO bioavailability gradually prevail. Increasing accumulation of AGE may be one of the culprits in this process. In addition, this balance is continuously modified by actual metabolic control and the degree of insulinopenia.

Alterations of the NO system in the diabetic kidney and their role in the pathophysiology of diabetic nephropathy still represent a great challenge for future research. There are a number of topics in this area that warrant further investigation. Future investigations in this area may focus on, among other topics, direct in vivo measurements of NO production in different compartments of the diabetic kidney and their changes in response to various stimuli; the biochemistry of eNOS and nNOS with respect to the changes in quaternary structure and cellular localization and posttranslational changes; and activity of signaling pathways, leading to modulation of NOS activities, as well as on possible alterations in NO signaling. Considering the epidemic increase in type 2 diabetes-associated nephropathy, studies should focus on evaluating these systems in models of type 2 diabetes.
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