Role of Nitric Oxide in Diabetic Nephropathy

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Diabetic nephropathy is the leading cause of end-stage renal disease in the Western hemisphere. Endothelial dysfunction is the central pathophysiologic denominator for all cardiovascular complications of diabetes including nephropathy. Abnormalities of nitric oxide (NO) production modulate renal structure and function in diabetes but, despite the vast literature, major gaps exist in our understanding in this field because the published studies mostly are confusing and contradictory. In this review, we attempt to review the existing literature, discuss the controversies, and reach some general conclusions as to the role of NO production in the diabetic kidney. The complex metabolic milieu in diabetes triggers several pathophysiologic mechanisms that simultaneously stimulate and suppress NO production. The net effect on renal NO production depends on the mechanisms that prevail in a given stage of the disease. Based on the current evidence, it is reasonable to conclude that early nephropathy in diabetes is associated with increased intrarenal NO production mediated primarily by constitutively released NO (endothelial nitric oxide synthase [eNOS] and neuronal nitric oxide synthase [nNOS]). The enhanced NO production may contribute to hyperfiltration and microalbuminuria that characterizes early diabetic nephropathy. On the other hand, a majority of the studies indicate that advanced nephropathy leading to severe proteinuria, declining renal function, and hypertension is associated with a state of progressive NO deficiency. Several factors including hyperglycemia, advanced glycosylation end products, increased oxidant stress, as well as activation of protein kinase C and transforming growth factor (TGF)-β contribute to decreased NO production and/or availability. These effects are mediated through multiple mechanisms such as glucose quenching, and inhibition and/or posttranslational modification of NOS activity of both endothelial and inducible isoforms. Finally, genetic polymorphisms of the NOS enzyme also may play a role in the NO abnormalities that contribute to the development and progression of diabetic nephropathy.
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$\mathbf{T}$HE INCIDENCE and prevalence of diabetes is increasing worldwide at an alarming rate so that many consider it a global epidemic. Diabetic nephropathy is a major microvascular complication of diabetes and progression to end-stage renal disease (ESRD) almost always is inevitable unless preventive strategies are implemented in the very initial stages of nephropathy. Diabetes as a cause of ESRD has surpassed all other diseases, accounting for approximately 40% of all cases of ESRD in the United States. Currently, diabetic nephropathy is incriminated as the leading cause of ESRD not only in the United States but in the entire Western hemisphere and is emerging as one of the major diseases resulting in ESRD even among developing nations. Although the reasons for the global epidemic of diabetes mellitus remain an enigma, new insights into pathogenesis and therapeutic strategies continue to emerge that potentially might make a difference in this devastating medical problem.

Several factors have been incriminated in the pathogenesis of diabetic renal disease. Uncontrolled hyperglycemia, systemic and intrarenal hypertension, hyperlipidemia, and activation of renin-angiotensin are the most important among those factors. Previous studies suggested that chronic inhibition of nitric oxide (NO) played a role in the development and progression of chronic renal failure in diabetes.[1,2] The synthesis of NO in the kidney and its role in renal functions is discussed in another section of this issue. Briefly, NO is an inert gas, with an extremely short life that is synthesized from its sole precursor, L-arginine, through the action of nitric oxide synthase (NOS), of which there are 3 isoforms. These include neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III), type I and II being expressed constitutively and all 3 isoforms have been shown to exist in the kidney. Alterations in intrarenal NO synthesis theoretically may play an important role in the aforementioned as well as other pathogenetic factors of diabetic nephropathy. This article reviews the current evidence from experimental and clinical studies that support the pathogenetic role of abnormalities of NO in the development and progression of diabetic nephropathy.

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Diabetic nephropathy is diagnosed clinically by the onset of proteinuria that usually is determined by dipstick detection of protein in a random urine sample. However, a long period of subclinical microalbuminuria, often lasting years, precedes the clinical diabetic nephropathy during which glomerular hyperfiltration and hypertrophy are evident. Several pathogenic mechanisms are incriminated in this early phase of nephropathy including activation of the renin-angiotensin system. With increasing proteinuria, nephropathy progresses to an advanced clinical state characterized by hypertension, renal failure, and glomerulosclerosis. In the past decade, innumerable studies have accumulated in the literature that examined the role of NO in diabetic kidney. However, these studies, which were both in vitro and in vivo models, have expanded our understanding of the interaction of the diabetic state with intrarenal NO and more specifically the role of NO in the pathophysiology of diabetic nephropathy. But these studies unfortunately also significantly added to the confusion that exists regarding this subject, in view of contradictory findings reported. This article aims to outline salient studies that support the evidence for the role of NO in diabetic kidney disease and also point out some of the controversies in this field.

RENAL NO SYSTEM IN EXPERIMENTAL DIABETIC MODELS

Experimental studies that examined the effect of diabetes on the intrarenal NO system included in vivo studies as well as in vitro studies in the cell culture and isolated kidney models. The in vitro models particularly dealt with effects of glucose and other biochemical sequelae of the diabetic state such as advanced glycation end products (AGE) and activation of angiotensin II. Although most in vitro models suggested a depressed renal NO system or NO availability, most in vivo models showed enhanced NO production.

NO in Diabetes Mellitus: In Vitro Models

Hyperglycemia is considered a major pathophysiologic mediator of renal injury in diabetic nephropathy.[3] Most studies that examined the effects of high ambient concentrations of glucose on the kidney in vitro including cell cultures,[4,5] glomerular explants,[6,7] and renal vasculature,[8,9] and isolated kidneys in vitro[10] showed that NO production is impaired significantly in such models. Cell culture studies examining the effects of glucose have reported impaired NO production or decreased NO bioavailability. Several mechanisms have been shown or hypothesized to mediate this effect. Mesangial cells cultured in high glucose showed decreased inducible NO production (NOS II) or availability,[11,12] although enhanced production was shown by other investigators.[13,14] However, in all these studies, iNOS messenger RNA and protein were unaffected by high glucose levels. Studies from our laboratory[12] have shown that high glucose levels resulted in a posttranslational inhibition of mesangial iNOS owing to decreased stability and hence availability of tetrahydrobiopterin (BH_4), a cofactor for NOS enzyme (Figs 1A and 1B). Other mechanisms that have been offered to explain the inhibition of NO activity by high glucose levels include activation of protein kinase C,[6,7,15] glucose quenching of NO,[16] and inhibition of NOS activity,[4,5] with generation of reactive oxygen species diminishing the NO availability.[15] Recent studies by Trachtman et al[17] showed that although glucose directly inhibited mesangial NO production, growth factors such as insulin-like growth factor-1, and epidermal growth factor opposed this effect of high glucose, suggesting that some peptide growth factors modulate the mesangial NO production in the diabetic state. However, the same investigators found no such modulation of high glucose effects on mesangial NO synthesis by vascular endothelial growth factor.[18] Other investigators found that although incubation of glomerular endothelial cells in 30 mmol/L glucose resulted in a rapid increase in NO release in periods up to 6 hours, exposure beyond 12 hours blunted the NOS activity in the cells[19] compared with cells grown in standard (5 mmol/L) glucose.

Glycosylation of proteins and formation of AGE is a consequence of long-term exposure of tissue proteins to high glucose concentrations and may contribute to decreased renal NO synthesis and/or availability. Some studies have shown that formation of AGE in rat glomeruli was associated with increased nitrites and nitrates (NOx) in the urine,[21] and increased iNOS expression[20] and eNOS activity. However, most studies[22] have confirmed that AGE accumulation resulted in reduced NO levels by several mechanisms including increased degradation of eNOS messenger RNA,[23] decreased eNOS protein expression,[24,25] decreased eNOS activity,[4] quenching and/or inactivation of NO,[26,27]
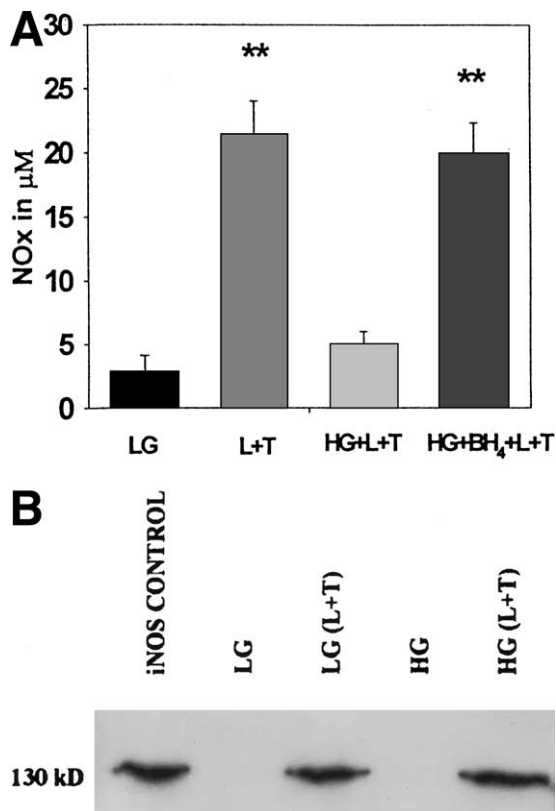
Fig 1. (A) Effects of tetrahydrobiopterin (BH$_4$) on NOx accumulation in MMC. **$P < .001$. L + T versus LG and HG + BH$_4$ + L + T versus HG + L + T, $n = 12$ in each group. (B) Western blotting showing effects of HG on iNOS protein expression. The immunoblot represents similar results from 3 other experiments. Densitometric image analysis revealed no significant differences between the bands seen in lane LG + (L + T) and lane HG + (L + T). Reprinted with permission from Prabhakar SS: Tetrahydrobiopterin reverses the inhibition of nitric oxide by high glucose in cultured murine mesangial cells. Am J Physiol 281:F179-F188, 2001.[12]

and increased oxidative stress.[28] In a cell culture model, AGEs were found to block the cytostatic effect of NO on aortic smooth muscle and renal mesangial cells[27] and based on such findings Hogan et al argued that NO inactivation and consequent blockade of antiproliferative effects of NO by AGE may be a common pathway in the development of accelerated renal and vascular disease in diabetes. Studies from our laboratory have shown that long-term exposure of mesangial cells resulted in inhibition of iNOS-derived NO, an effect that was reversed partially in the presence of an inhibitor of AGE formation 2,3 diamno-phenazine (un-

published observations). This effect was associated with a reduction in the formation of peroxynitrite, indicating that AGE-induced NO inhibition was related partly to increased formation of reactive oxygen species.

NO in Diabetes Mellitus In Vivo Models

NOS Isoforms in Diabetic Kidney

Extensive literature documented the significance of NO and NOS expression in in vivo studies of diabetic animal models. Although some studies have shown increased expression of all isoforms of NOS in diabetic kidneys,[29] most studies have shown increased eNOS expression[21,30,31] and eNOS activity,[32,33] especially in kidneys from the early stages of diabetes. Similar observations were made in streptozotocin-induced diabetic rats with the in vivo hydronephrotic kidney technique.[30] In this study, the investigators showed that urinary NOx was increased along with increased eNOS expression in the total kidney (immunocytochemistry) and in isolated renal arteries (Western blotting). Other studies have shown that eNOS and nNOS expression is decreased in diabetic kidneys.[34,35] Taken together the aforementioned studies strongly indicated increased eNOS activity with augmented basal NO release, which might explain intrarenal vasodilation and hyperfiltration seen in early diabetes. With reference to nNOS (NOS I), decreased levels of urinary NOx along with decreased nNOS expression were found in kidneys of streptozotocin diabetic rats that were not treated with insulin.[34] These changes were noted 30 hours after the induction of diabetes, with reduction in total NOS activity of renal cortex and diminished immunocytochemical expression of nNOS and eNOS in the kidney. Furthermore, some studies have shown reduced iNOS expression[31,36] or unchanged iNOS[32,37] in diabetic kidneys. Thus, it seems reasonable to summarize that eNOS expression is increased especially in early diabetes, and nNOS and iNOS are expressed variably in diabetic kidneys.

To explain the decreased eNOS activity and eNOS expression in hyperfiltering glomeruli observed in some studies, the activity of nNOS was examined by some investigators.[38] By using S-methyl-L-thiocitrulline, a nNOS-specific blocker, they showed a stronger renal vasoconstriction in diabetic rats than in control rats and addition of the

nonspecific blocker L-NAME did not affect the glomerular filtration rate (GFR) any further. Additionally, S-methyl-L-thiocitrulline–treated moderately hyperglycemic streptozotocin diabetic rats had a higher GFR along with increased nNOS positive cells compared with control rats.[39] Based on these data, they argued that nNOS might be the major isoform of NOS involved in mediating hyperfiltration of early diabetes. Other studies supported this argument as well.[37,40]

The significance of iNOS in diabetic kidneys was overlooked and its importance was disregarded in some recent reviews.[41] Early studies showed increased expression of iNOS, mediated by tumor necrosis factor-α in diabetic rat glomeruli at 52 weeks after diabetes induction by streptozotocin.[36] The investigators correlated this effect to AGE formation because aminoguanidine reversed these changes. A similar increase in iNOS expression in the kidney was seen in rats fed with a high-fructose diet, which was associated with increased glomerular size and filtration rate.[42] These changes were prevented by simultaneous administration of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). Because aminoguanidine is also a potent iNOS-specific inhibitor, it had been argued often that the renoprotective benefits of aminoguanidine in diabetic nephropathy were due to iNOS inhibition rather than to AGE inhibition. However, this view was shown to be incorrect in a study performed specifically to address this question.[43] On the other hand, there are several recent studies that documented that exposure to high glucose impaired iNOS activation in mesangial[11,12] and vascular smooth muscle and endothelial cells.[44-46] Decreased iNOS expression in renal cortex was described in diabetic rats.[31] Furthermore, AGE formation has been shown to inhibit iNOS in proximal tubular cells[4] and mesangial cells (Prabhakar, unpublished observations). Recently, Trachtman et al[47] examined the role of iNOS in C57BL/6 and iNOS knockout mice with a streptozotocin-induced diabetic state. After 40 weeks of a diabetic state without insulin both mice had similar degrees of proteinuria, serum creatinine levels, and renal hypertrophy. Both groups showed decreased expression of iNOS and increased eNOS in the kidneys by immunostaining. However, knockout mice had decreased urinary NOx, increased tubulointerstitial fibrosis, and mesangial expansion than C57BL/6 mice. These data suggest that iNOS-

derived NO modulated glomerulosclerosis in chronic streptozotocin diabetic nephropathy and a lack or inhibition of iNOS accelerated diabetic nephropathy. Thus, most studies that examined iNOS in diabetic milieu found inhibition of expression or activity of the enzyme in in vitro studies.

Intrarenal NO and Renal Hemodynamics

The increased expression and activity of eNOS in the diabetic state is believed by some investigators to explain the functional and glomerular hemodynamic changes associated with early diabetic nephropathy. These include increased renal plasma flow, hyperfiltration, and filtration fraction. Evidence for such hypothesis was sought by in vivo studies examining the effects of general and isoform-specific NOS inhibitors on renal hemodynamics. The pilot observation that the NOS inhibitor L-NAME resulted in a greater vasoconstriction in renal vasculature of diabetic rats than in control rats was made in Brenner's laboratory,[48] suggesting a strong influence of renal NO levels on renal and glomerular hemodynamics. This finding was followed by a series of publications that suggested that the hyperfiltration of early diabetes was associated with increased vasodilatation of renal microvasculature and increased NO synthesis as reflected by urinary NOx.[49-51] The concept was consolidated further by observations from other studies that showed that vasodilation induced by acetylcholine, an NO-mediated process, was amplified in diabetic kidneys[33] and that L-NAME significantly decreased glomerular hyperfiltration.[21,31,52]

However, many other studies did not support this concept[53-55] and showed that the vascular responses to NO or L-NAME were rather blunted with decreased urinary NOx, suggesting that renal NO production is defective and contributed to renal injury in diabetes. By using videomicroscopic studies, Pflueger et al[56] showed that responses to L-NMMA and L-arginine were blunted in diabetic kidneys, particularly in the cortex, indicating that renal NO production is impaired in diabetes. By using acetylcholine infusion, an eNOS-specific agonist, Wang et al[57] showed that vascular responses to acetylcholine were impaired in diabetic rats, which were not restored with insulin treatment. These data underscore the importance of diminished bioavailability of NO in diabetic renal vas-

culature and defects in receptor-mediated eNOS activation.

Several studies have suggested that long-term modulation of the renal NO system may influence the progression of diabetic nephropathy. Most such studies indicated that NO might play a renoprotective role in the course of diabetic nephropathy. Chronic NOS inhibition was shown in earlier studies to be associated with renal structural and functional damage.[58,59] Because the kidney is the principal source of L-arginine levels in plasma, decreased arginine synthesis in chronic renal failure, especially if dietary arginine content is limited, might result in arginine deficiency and contribute to a NO-deficient state. The study by Reyes et al[60] showed that long-term administration of L-arginine decreased proteinuria and hyperfiltration in diabetic rats. Although there were flaws in this study, Lubec et al[61] have shown similar findings in diabetic kk mice. Other studies[58] showed that treatment with L-NAME, a NOS blocker, accelerated proteinuria and was associated with higher levels of transforming growth factor (TGF)-β, implicating the latter in diabetic nephropathy. Furthermore, Fujihara et al[62] had shown similar findings in a nephrectomy model of renal failure in nondiabetic rats, in which L-NAME worsened proteinuria and glomerular injury, but these changes were ameliorated with angiotensin blockade with losartan. The beneficial role of L-arginine in diabetic nephropathy was discussed previously in a review by Klahr[63] and in a separate review in this issue.

HUMAN STUDIES

As opposed to in vitro and animal studies, clinical studies examining NO production in diabetic subjects are limited, although rapidly increasing in recent years. Earlier studies have shown increased urinary NOx in normo-albuminuric diabetic subjects with increased GFR compared with nondiabetic healthy controls and this change was associated with increased immunostaining for eNOS (NOS III) in the glomeruli.[64] These data suggested that increased NO produced by eNOS might be involved in hyperfiltration of early diabetic nephropathy. Dalla Vestra et al[65] have shown that infusion of L-NAME into type 2 diabetic subjects did not decrease the GFR and filtration fraction as in control nondiabetic subjects, whereas the cardiac index was decreased in all the groups. The

investigators concluded that modulation of glomerular hemodynamics is different and independent from NO-regulated cardiac effects. However, other studies indicated that patients with type II diabetes and microalbuminuria have decreased NO production as measured by stable NO metabolites in the urine of the patients.[66] By using a new high-performance liquid chromatography–Greiss method of measuring NOx, Maejima et al[67] showed that plasma NO availability was impaired in diabetics with advanced microvascular complications and the decreased NO bioavailability correlated with AGE formation and reactive oxygen species. Defective endothelial-dependent vasodilatation in diabetic nephropathy subjects was established several years ago.[68] Duplex sonographic studies in patients with diabetic nephropathy have shown reduced renal vasodilatory response to nitroglycerine, a NO donor, compared with healthy controls.[69] These data suggested that altered renal hemodynamic responses in diabetic nephropathy were mediated by impaired NO release or availability. Decreased NO availability also may be due to conversion of NO to peroxynitrite ($ONOO^-$) in the presence of superoxide (O_2^-) in conditions of uncontrolled hyperglycemia. Subsequently, $ONOO^-$ nitrosylates tyrosine residues on protein molecules, resulting in tissue injury. Recent studies confirmed that in renal biopsy specimens from diabetic patients, immunostaining for nitrotyrosines is increased compared with normal kidneys and is particularly evident in proximal tubules and in loop of Henle.[70] These data support the concept of decreased NO bioavailability consequent to increased formation of reactive oxidative species in diabetic kidneys. Human studies examining the role of eNOS gene polymorphisms in diabetic nephropathy have been controversial. The allele of intron 4 of the eNOS gene has been reported to be more frequent in dialysis-dependent diabetics compared with diabetics with no renal disease.[71] Similar results were reported in type 2 diabetic nephropathy subjects from Japan.[72] On the other hand, other studies reported such associations in nondiabetic renal disease but not in diabetic nephropathy.[73]

EFFECTS OF OTHER FACTORS IN DIABETIC MILIEU ON RENAL NO SYSTEM

Angiotensin

The diabetic state is associated with activation of the intrarenal renin angiotensin system. The

therapeutic success of angiotensin blockade with angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) in retarding the progression of diabetic nephropathy testifies the pathophysiologic significance of angiotensin activation in diabetic kidney disease. The effects of such blockade on renal NO synthesis and if such effects mediate the beneficial consequences seen with ACE inhibition and ARB in diabetic nephropathy have been the subject of investigation of several studies. The evidence is conflicting about the angiotensin effects on renal NO synthesis. Angiotensin has been shown to increase NO production in proximal tubular cells,[74] renal cortex,[75,76] renal medulla,[77] and isolated renal arteries.[78] In all these studies, it was argued that enhanced renal production is a defense mechanism against the detrimental effects of angiotensin. However, several recent studies, including one from our own laboratory, have shown that angiotensin II inhibited NO synthesis in different renal cells in culture.[79-81] Although the isoform involved in all of these studies was iNOS (NOS II), constitutively expressed NOS was involved in the studies in which angiotensin stimulated NO synthesis. Furthermore, data from our laboratory (unpublished observations) and other studies[79] indicated that angiotensin alone in the absence of cytokines had no effect on mesangial or renal NO synthesis. It appears reasonable to conclude that angiotensin may have stimulatory effects on constitutive NOS, but in the presence of cytokines that often are activated in the diabetic milieu, angiotensin may exert an inhibitory effect on iNOS, and probably the whole kidney NO production. Results from our laboratory indicating that losartan enhanced NO production in mesangial cells concur with observations of Fujihara et al,[62] who showed that angiotensin inhibition with losartan slowed renal injury functionally and structurally in a renoprival model of renal failure. An additional mechanism by which angiotensin II impairs NO availability is through increased oxidative stress. Wilcox et al[82] showed that early diabetic proteinuric nephropathy was associated with increased expression of p47phox component of the reduced form of nicotinamide-adenine dinucleotide phosphate oxidase and eNOS with increased H_2O_2 formation in the kidney and treatment with an ACE inhibitor or ARB reversed these changes with amelioration of proteinuria. These findings confirm the strong angiotensin-NO interactions in the kidney and potential implications in diabetic nephropathy. Furthermore, Forbes et al[83] showed that both ramipril (an ACE inhibitor) and aminoguanidine decreased AGE formation in diabetic rat kidneys as determined by immunohistochemistry and fluorescence and these changes correlated with reduction in nitrotyrosine accumulation in the kidney. Based on these observations, the investigators proposed a linkage between AGE and the renin-angiotensin system in diabetic nephropathy, mediated by oxidative stress.

Insulin

Hyperinsulinemia is an important and independent risk factor for the cardiovascular complications of diabetes. Hyperinsulinemia is seen not only in type II but also in type I diabetes and is incriminated in the development of hypertension and cardiovascular risk in such patients. However, the effects of insulin per se on renal NO synthesis is far from clear. Insulin in the physiologic range stimulates NO release from the kidney[84] whereas hyperinsulinemia was noted to inhibit renal excretion of NO metabolites in the urine.[85] We recently have shown that insulin in physiologic concentrations enhanced iNOS activity and NO synthesis in normal human mesangial cells and further that the augmented cellular uptake of L-arginine mediated this effect of NO production.[86] However, in the clinical state of advanced diabetic nephropathy, it is very likely that the cellular resistance to the effects of insulin prevents the augmentation of NO synthesis despite hyperinsulinemia. NO regulates the release of insulin from pancreas[87] but the role of different isoforms in the regulation of insulin release is not well understood. Studies including those from our laboratory have shown that NO released from both constitutive and iNOS mediate high glucose-stimulated insulin release from islet β pancreatic β cells. However, studies from our laboratory first noted that at maximal activity of iNOS, the cytopathic effects of cytokines on pancreatic islet cells result in a paradoxic decrease in insulin secretion.[88] Furthermore, recent observations from our laboratory show that enhanced insulin secretion seen with ACE inhibition and ARB is mediated by augmented NO release from islet beta cells.

Protein Kinase C

That diabetes mellitus is associated with activation of protein kinase C is well established.[89,90] The activity of NOS, particularly eNOS, is regulated by highly harmonized phosphorylation and dephosphorylation of serine, threonine residues of the eNOS enzyme. Protein kinase C phosphorylates Threonine 495 and dephosphorylates Serine 177 residues of the eNOS enzyme, thereby inhibiting eNOS catalytic activity.[91] These data provide an explanation for the widely believed concept that advanced diabetic nephropathy is a NO-deficient state.

TGF-β

Diabetic metabolic milieu is associated with activation of TGF-β in the kidney[92] and such activation has been shown to mediate mesangial expansion and extracellular matrix expansion in human and experimental diabetic nephropathy.[93] Angiotensin[94] and hyperglycemia have been incriminated in activation of TGF-β in diabetic kidney. In fact, several metabolic and pathophysiologic consequences of angiotensin II and hyperglycemia are believed to be mediated by activation of TGF-β. High glucose–induced activation of TGF-β was shown to be mediated through increased thrombospondin-1 expression, which in turn is regulated by a NO–cyclic guanosine monophosphate–dependent protein signaling pathway.[95] We recently have shown that in the presence of angiotensin, inducible NO production from cytokine-stimulated mesangial cells was blocked and this was associated with increased TGF-β expression shown by immunocytochemistry. Furthermore, the inhibitory effects of angiotensin on iNOS were reversed in the presence of anti-TGF antibodies (Figs 2A, 2B, and 2C). These data from our laboratory and others[96] support the hypothesis that TGF-β may modulate the pathophysiologic phenomena in diabetic nephropathy and the therapeutic effects of angiotensin blockade may involve modulation of TGF-β activity in the kidney.
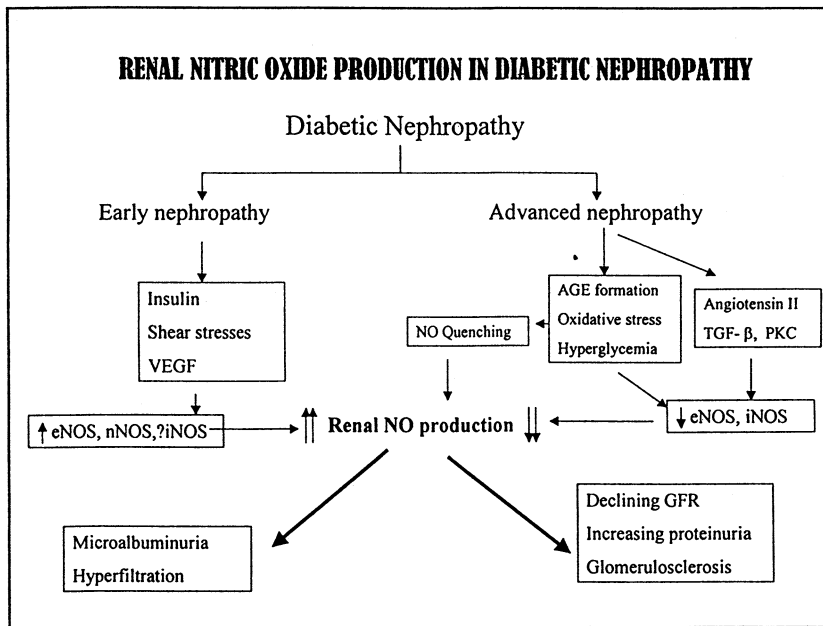
GENETIC POLYMORPHISMS OF NOS AND DIABETIC NEPHROPATHY

Susceptibility to diabetic nephropathy mostly is determined genetically. Endothelial dysfunction is the pathophysiologic denominator of vascular complications of diabetes including nephropathy.
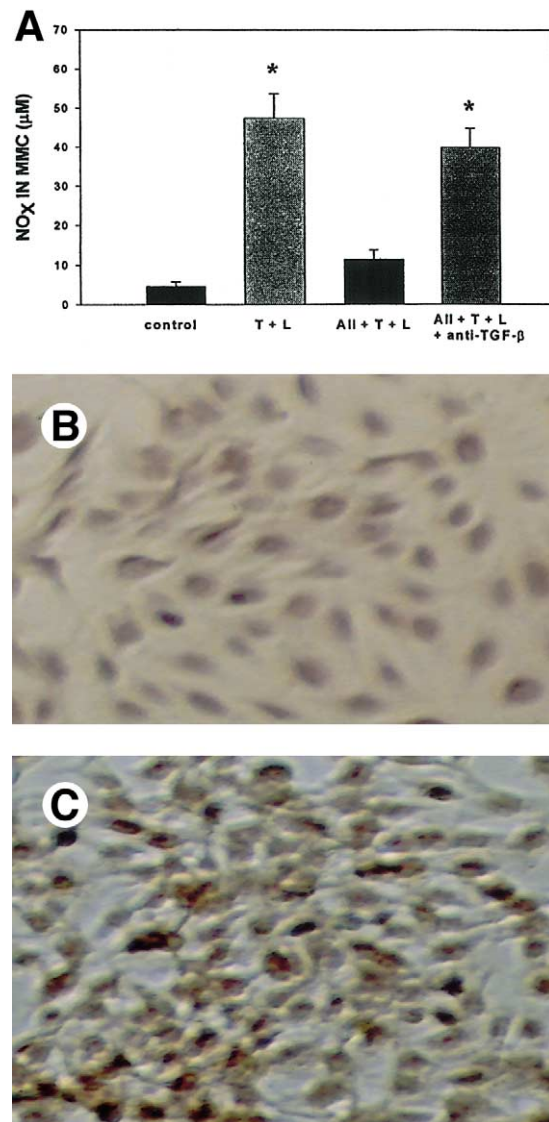
Fig 2. (A) Effects of angiotensin II on NO production in mouse mesangial cells and mediation through TGF-β. T, tumor necrosis factor-α, L, bacterial lipopolysaccharide, NOx, nitrites and nitrates in the media. *P < .01 versus control, n = 12 in all groups. (B, C) Immunocytochemical expression of TGF-β in mouse mesangial cells stimulated with tumor necrosis factor-α and bacterial lipopolysaccharide in the (B) absence and (C) presence of angiotensin II.

Because NO regulates endothelial function it is imperative to conceptualize that abnormalities of the NOS gene would influence the development of diabetic nephropathy. Indeed, several studies were published that examined the association of NOS gene polymorphisms, especially the eNOS isoform, with susceptibility to diabetic nephropathy.

RENAL NITRIC OXIDE PRODUCTION IN DIABETIC NEPHROPATHY

Diabetic Nephropathy

Early nephropathy   Advanced nephropathy

Insulin
Shear stresses
VEGF

NO Quenching

AGE formation
Oxidative stress
Hyperglycemia

Angiotensin II
TGF- β, PKC

↑eNOS, nNOS,?iNOS → ⇈ Renal NO production ⇊ ← ↓eNOS, iNOS

Microalbuminuria
Hyperfiltration

Declining GFR
Increasing proteinuria
Glomerulosclerosis

Fig 3. Schematic representation of NO changes in early and advanced diabetic nephropathy and various pathophysiologic triggers for such changes.

Specifically, Glu298Asp mutation at exon 7 and a 27-bp variable number tandem repeat in intron 4 insertion/deletion polymorphism of the eNOS gene were evaluated in the majority of these studies. Interestingly, similar to the preceding sections, literature is confusing with controversial findings and evidence both supporting and disputing the role of genetic polymorphisms in diabetic nephropathy. Most studies that examined the prevalence of eNOS genetic polymorphisms compared diabetics with nephropathy (advanced chronic renal failure or proteinuria) with diabetics with no renal disease (control). Although a significant association was described in some studies,[72,97-101] other studies have reported no such association with progression of nephropathy in type 2 diabetes.[102-105] Similar associations were described to affect the progression of nephropathy in type I diabetes[106] and nondiabetic renal disease.[72] On the other hand, a recent study has disputed the association of NOSIII gene polymorphisms in type I diabetes with renal disease.[104] Some other studies that examined the NOS-II gene in type I diabetes, particularly CCTTT-repeat polymorphisms, reported such variations confer protection from development of diabetic nephropathy.[107] Although these data from the earlier-cited conflicting studies may not be reconciled easily without incriminating differences in the type of patient populations studied, stage of

renal failure, and so forth, more systematic and large-scale population studies are needed to confirm such genetic associations in diabetic nephropathy.

SUMMARY

The literature pertaining to the role of NO abnormalities in diabetic nephropathy is vast but composed of confusing and conflicting data. The controversies stem from several issues that involve different models studied, various NOS isoforms and species studied, stage of renal disease in diabetes, and methodologies used, and many more. The in vitro studies, though being very valuable in understanding the pathophysiology, do not reflect the net effect of coordinating and regulatory influences of several signaling systems that act in concert with each other as in whole animal or in vivo studies. However, despite these differences, some general patterns seem to emerge when the data are analyzed carefully. Diabetic metabolic milieu triggers a variety of autocrine and paracrine mechanisms that modulate the renal NO system, both stimulatory and inhibitory signals acting in parallel. The net influence on renal NO availability depends on the balance between these opposing influences. Most evidence suggests that during the early phases of hyperfiltration and increased GFR,

the net result of these opposing signals is to augment renal NO production, often contributing to these glomerular hemodynamic changes. This effect is mediated by activation of constitutively released NO, mediated by eNOS and to a lesser extent nNOS. As the nephropathy progresses in diabetes with increasing proteinuria and decreasing renal function, a state of renal NO deficiency sets in, which results from decreased production and/or decreased availability of NO as a consequence of multiple mechanisms (Fig 3). In general, insulinopenia and the degree and duration of impaired metabolic control are the major factors that facilitate mechanisms that suppress NO availability to prevail in advanced diabetic nephropathy. Such mechanisms include decreased activity of NOS isoforms, often as a result of activation of protein kinase C, high ambient glucose levels, or decreased cofactor availability (eg, BH$_4$), activation of TGF-β both by high glucose and angiotensin II levels, increased formation of NOS inhibitors such as asymmetric dimethyl arginine, and reactive oxygen species often secondary to angiotensin and AGE effects. Thus, gradual accumulation of AGE and induction of plasminogen activator inhibitor-1 lead to decreased eNOS activity and reduced NO production resulting in endothelial dysfunction.[24] Evidence suggests that NO from all NOS isoforms may be depressed including iNOS, which often has been considered insignificant hitherto. The role of genetic polymorphisms of NOS enzymes and decreased arginine in the kidney as a contributor to NO deficiency in advanced diabetic nephropathy is appealing but needs further confirmation.

The role of the renal NO system in the physiology of the kidney and specifically in the pathophysiology of diabetic nephropathy remains a complex area with conflicting observations and constitutes a very challenging and fertile field for future investigation. Specific questions that need to be investigated include signaling mechanisms that influence renal NO production, direct in vivo assessment of NO changes in the diabetic kidney, and the role of genetic factors that influence renal NO generation. The global epidemic of diabetes warrants concerted efforts to decipher the mechanistic pathways leading to diabetic vascular complications, which would in turn facilitate discovery of novel therapeutic interventions.

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