

eNOS and Diabetic Nephropathy

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Abstract

Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD) and the number of patients with DN has been increasing rapidly. Development of new effective therapeutic strategies of DN is slow at least partly because of a lack of animal models that recapitulate the features of human DN. Human variants of the endothelial nitric oxide synthase gene (eNOS, NOS3) that produce reduced amounts of nitric oxide (NO) are positively associated with DN, although proof of causation is lacking. Recently, several investigators have established animal models of advanced DN using mice unable to synthesize eNOS and have demonstrated that eNOS ^{-/-} mice with diabetes develop severe nephropathy. It has been also shown that a high fat diet worsens the DN of the diabetic mice lacking eNOS. However, complete absence of eNOS has not been reported in humans, although reduced levels are not infrequent. Accordingly, heterozygous eNOS ^{+/-} mice have been made diabetic and they demonstrated that the decrease in eNOS/NO comparable to that of NOS3 polymorphisms is sufficient to cause exacerbation of DN. Increased expression of tissue factor (TF), initiator of coagulation, plays a significant role in the DN of the eNOS ^{-/-} diabetic mice, as well as in diabetic mice having wild type eNOS. Strategies to ameliorate hypercoagulability could be useful for treatment of DN.

Keywords: Mouse; Tissue factor; Inflammation

Introduction

More than 30% of all diabetic patients develop diabetic nephropathy (DN) [1], which is the most frequent cause of chronic kidney disease (CKD) and is a risk factor for stroke and heart attack [2]. The number of diabetic patients and those affected by DN is rapidly increasing [3]. Not all diabetic patients develop DN and the development and severity of DN vary greatly from one patient to another with familial clustering, suggesting that genetic factors play an important role [4]. One of the most well-known changes in genes that exacerbate DN is increased expression of angiotensin converting enzyme (ACE) [5]. Recently, genome wide association studies (GWAS) have identified several susceptibility loci associated with DN [6]. Although all these genetic and epigenetic studies suggest the association between the genes and DN, they do not prove the causality. Different from human studies, animal studies are very informative because their genetic background and environmental factors such as diet can be controlled. It is becoming more and more important to establish animal models, which recapitulate human DN, where causation can be explored.

Increased expression of ACE exacerbates DN in mice [7], indicating that increased ACE expression as in ACE DD genotype causes an exacerbation of DN. Although ACE inhibitors are very effective anti-hypertensive drugs, mice with mildly increased ACE comparable to individuals with the ACE DD genotype do not have increased blood pressure (BP) or plasma angiotensin II (Ang II) levels [8], indicating that the exacerbation of DN caused by a mild increase in ACE is not due to increased Ang II (Figure 1A).

The resolution of this contradiction is illustrated by the computer simulation shown in Figure 1. The simulation showed that mild inhibition of ACE, as in the *Ace* one-copy mice (equivalent to 50% ACE inhibition), causes an increase in Ang I, which offsets the decrease in ACE so that plasma Ang II and BP stay normal. However, if ACE is extensively inhibited, plasma Ang I levels plateau and further decreases in ACE causes a decrease in Ang II and BP. Mild changes in the expression of ACE, such as those seen in the human polymorphism and in the mouse gene titration experiments, are not sufficient to move

the system away from the offset region to where ACE differences affect Ang II levels and BP. ACE inhibitors (ACEI) on the other hand move the system to the region where Ang II levels and BP are decreased. In addition to converting Ang I to Ang II, ACE inactivates bradykinin (BK) and mild increase in ACE comparable to ACE DD genotype decreases BK by 20% in mice, suggesting that advanced DN by mild increase in ACE is likely due to a decrease in BK levels (Figure 1A) [8]. This hypothesis has been confirmed in experiments that diabetic mice lacking type 2 bradykinin receptor (B2R) exhibit accelerated DN compared to wild type diabetic mice [9].

Animal models of Diabetic Complications Consortium (AMDCC), currently Diabetic Complications Consortium (DCC, <http://www.diacc.org/>) was set up to develop good animal models of human DN, to investigate in mice the strain differences in DN and to investigate genes involved in DN [10,11]. The roles of bradykinin receptors were further investigated as part of AMDCC. Mice lacking both B1 and B2 receptors show severe DN [12,13]. Since stimulating bradykinin receptors activates eNOS, it is speculated that a decrease in eNOS activity also accelerates DN. Recently, multiple laboratories including ours have demonstrated that diabetic mice lacking eNOS develop human-like nephropathy [14-16].

NOS3 Polymorphisms and DN

NOS3 gene locates on chromosome 7q36, having 26 exons and 25 introns and is approximately 23.5kb in length. NOS3 polymorphisms leading to reduced NO production have been widely studied and are

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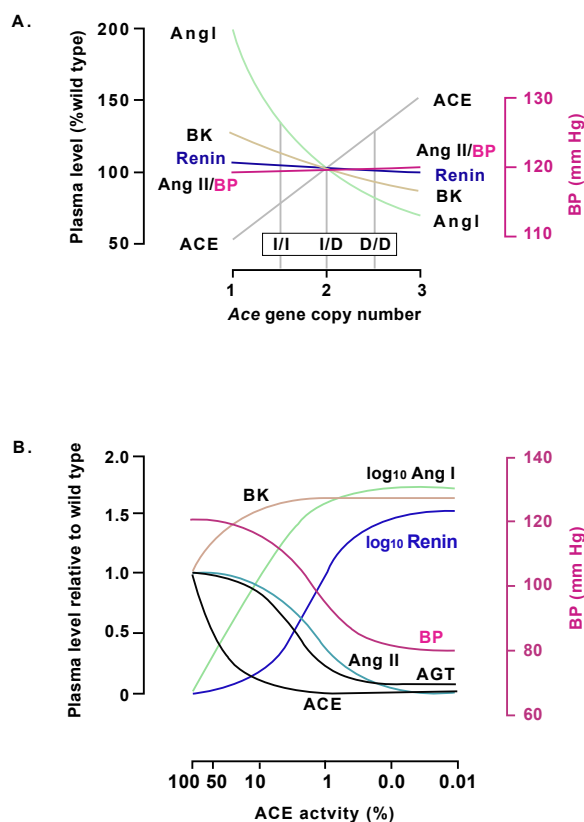


Figure 1: Simulations of Ace gene titration and progressive ACE inhibition. (A), ACE gene titration. Mild quantitative changes in the expression of ACE do not affect plasma Ang II level or BP because of the concomitant changes in the level of Ang I. However, plasma bradykinin (BK) level changes with changes in ACE expression. The levels expected in association with the human I/D polymorphism are shown. (B). Effects of ACE inhibition. When inhibition is virtually complete, the simulation shows that: 1) Ang I levels are high and plateaued; 2) Ang II levels are low; 3) BK levels are increased by the virtual absence of ACE; and 4) BP is substantially lowered. (Adopted from reference 8 with permission).

associated with a variety of diseases including essential hypertension [17], coronary artery disease [18] and venous thromboembolism [19]. To date, three common polymorphisms of NOS3 related to DN have been studied broadly. They are: 1) G894T in exon 7 that leads to the replacement of a glutamic acid by aspartic acid at residue 298 (Glu298Asp); 2) 4b/a with a 27 nucleotide variable number of tandem repeats within intron 4 (4a with four repeats and 4b with five repeats); 3) T-786C in the promoter region.

Association of the G894T (Glu298Asp) variant with DN

The 894T NOS3 gene produces about 30% of the amount of NO produced by the more common 894G [20]. Recent meta-analysis performed by Zeng et al. [21] show that this allele is associated with different effects in different populations. Total of 15 studies involving 6,725 individuals, about half of them are patients with DN, have been analyzed. In control groups, the frequency of 894T is higher in Caucasian than in East Asian. However, in Caucasian, there was no association between G894T (Glu298Asp) and DN. In East Asian, on the other hand, this polymorphism is positively associated with DN [21].

Association of the 4b/a variant with DN

The plasma level of nitrate/nitrite, the metabolites of NO, in individuals with the allele is 20% lower than that of non-carriers [22]. The relationship between the 4b/a variants and DN is uncertain. A meta-analysis carried out by Zeng et al. [21] showed that there was no association between the 4b/a variant and DN in global populations, although the association became significant if individuals with microalbuminuria were excluded from the study [21]. A meta-analysis performed by He et al. [23] likewise, did not find an association between 4b/a and DN in Caucasians, but their analysis showed a positive association in the global population and East Asian.

Association of the T-786C variant with DN

This mutation is associated with decreased promoter activity, eNOS transcription and NO production [24]. This allele of NOS3 has been shown to be associated with DN in global population. The meta-analysis performed by Zeng et al. [21] including 3,045 individuals (about 48% of them were diabetic patients with DN) showed that an overall association between T-786C and DN. Unlike two other variants, the positive association was also found in Caucasian when the analysis included only ethnic groups originated from Europe.

Features of human DN

Before discussing DN of mice lacking eNOS, we will briefly summarize the features of human DN.

Glomerular lesions: In the earliest stage of DN, glomerular hyperfiltration and hypertrophy develop in patients [25,26] and there is some association between early onset hyperfiltration, hypertrophy and the subsequent development of other pathological changes of DN [25-27]. Thickening of the glomerular basement membrane (GBM) is also one of the first measurable changes in diabetic patient 1.5–2.5 years after the onset of type I diabetes [28,29]. Thickening of GBM results from accumulation of extracellular matrix (ECM) proteins including collagen type IV, type V and fibronectin [25,30]. The accumulation of ECM also contributes to mesangial expansion [25], although initiation of GBM thickening and mesangial expansion occur independently at variable rates [26]. When mesangial expansion exceeds a Vv (volume of mesangium/volume of glomerulus) greater than 37%, patients will have some manifestation of nephropathy, such as microalbuminuria, decreased glomerular filtration rate (GFR) [31-33]. The progression of the mesangial lesions leads to Kimmelstiel-Wilson nodules [25] and the formation of Kimmelstiel-Wilson nodules is considered to indicate that DN is progressing to the next stage [25]. Together, these lesions finally result in glomerulosclerosis. The mechanisms underlying these pathological changes of DN are still largely unknown, although Nakagawa and we have respectively proposed “vascular endothelial growth factor (VEGF) -eNOS/NO uncoupling” and “Tissue Factor (TF)/hypercoagulability-inflammation” are involved, as discussed later in this review.

Tubulointerstitial lesions: Like thickening of GBM, thickening of the tubular basement membrane is due to excess ECM resulting from increased production and/or decreased degradation. This pathological change is detectable parallel to GBM thickening [34]. As DN proceeds, interstitial fibrosis and tubular atrophy also occur and glomerular lesions eventually lead to ESRD [35]. Presence of macrophages and T-lymphocytes in the interstitium [36] suggests that inflammation plays a role in tubulointerstitial lesions.

Vascular lesions: Hyalinosis of both afferent and efferent arterioles occurs within a few years after the onset of diabetes [37]. Stout et al. [38] suggest that hyalinosis of efferent arterioles is relatively specific for DN [25] and is a lesion which distinguishes DN from hypertensive nephropathy [25,39].

Mouse Models of DN: the Lessons from Diabetic eNOS -/- Mice

In general, the mouse is a relatively poor model of DN, only showing partial early stages of the lesions observed in human DN. In 2009, AMDCC proposed a validation criteria of mouse models of DN (<http://www.diacomp.org/>), including 1) functional changes: 50% or more decrease in GFR over the lifetime and ≥ 10 fold increase in urinary albumin excretion compared with controls with the same gender, age and strain. 2) structural changes: basement membrane thickening by electron microscopy; advanced mesangial matrix expansion with or without nodular sclerosis and mesangiolysis; tubulointerstitial fibrosis and any degree of arteriolar hyalinosis [11]. In the past five years, multiple laboratories have independently reported that advanced DN develops in mice lacking eNOS. The different groups have used different regimens to establish DN models in mice lacking eNOS and these models, to some extent, meet the criteria defined by AMDCC. Table 1 summarizes the features of DN of these models.

Low-dose STZ (streptozotocin), high fat (HF) diet regimen

We have initiated diabetes in male eNOS -/- and their littermate WT mice using low-dose STZ (40 mg/kg, 5 days) based on DCC protocol. Half of the mice were fed a HF diet. The combination of eNOS deficiency and HF diet causes severe pathological changes in the kidneys of diabetic mice, although their plasma glucose levels were not higher than those of WT diabetic mice. Diabetic eNOS -/- mice fed HF diet have increased urinary albumin excretion as early as 12 weeks after the onset of diabetes. At 24 weeks of diabetes, these mice show advanced DN, which meets almost all the criteria defined by DCC. Urinary albumin excretion increased to almost 5 times of WT diabetic mice and creatinine clearance was 50% of control mice, indicating progressive renal insufficiency. Strikingly, these mice show severe mesangiolysis and glomerulosclerosis (Figure 2A). They also have tubulointerstitial fibrosis, mesangial expansion and thickening of the GBM [14]. Kanetsuna et al. [40] also used the low-dose STZ regimen to investigate the role of eNOS/NO in DN using eNOS -/- mice with the same genetic background as ours [37]. Their mice were fed normal chow and their findings are consistent with ours.

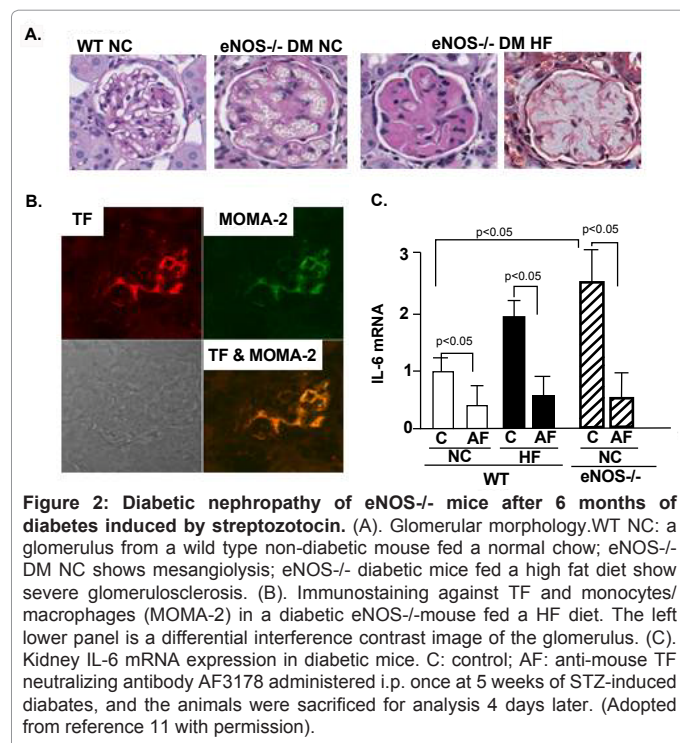
After establishing the mouse model of DN using STZ-diabetic eNOS -/- mice, we investigated the mechanism causing severe DN when eNOS is absent. Besides vasodilatation, eNOS/NO plays an important role against oxidative stress [41], inflammation [42] and coagulation [43]. Since micro-thrombi were found in glomeruli of diabetic eNOS -/- mice fed HF diet, it was hypothesized that activated coagulation system may be responsible for aggravating DN in these mice. Indeed, diabetic eNOS -/- mice fed HF diet had the most abundant fibrin deposits in their glomeruli, although glomeruli from all diabetic mice in this study were positive for fibrin. Kidneys from diabetic eNOS -/- mice have markedly increased TF expression and activity. Immunoreactive TF was almost exclusively expressed in macrophages/monocytes infiltrated in glomeruli (Figure 2B). Increased expression of TF precedes overt DN and administration of an anti-mouse TF neutralizing antibody to diabetic mice reduces the expression of inflammatory and fibrogenic genes, to a greater extent in eNOS -/- mice as shown, for instance, in Figure 2C. There are intricate relationships among eNOS/NO, TF and DN (Figure 3). NO inhibits [44] and HF stimulates [45] the NF- κ B pathway, one of the most important regulators of TF expression. Increased TF expression in the kidney activates the coagulation cascade, leading to the production of FVIIa, FXa and thrombin. These proteases can activate protease-activated receptors, which may enhance DN [46]. TF can also mediate inflammation. Interestingly, TF was only detected in monocytes/macrophages in glomerular mesangial area of our mice. Although immunoreactive TF was not detected in mesangial cells *in vivo*, mesangial cells are suggested to be responsible for mesangial expansion and glomerulosclerosis in DN. When macrophage in the mesangial area express TF and activate the coagulation cascade, FVIIa, FXa and thrombin will be produced, which could increase the expression of inflammatory genes including MCP-1 in mesangial cells, possibly through binding to protease activated receptors. Hypercoagulability and inflammation together likely make a vicious circle in exacerbating DN.

eNOS +/- Akita and eNOS -/- Akita mice

Although lack of eNOS exacerbates DN in mice, complete lack of gene products in general human population does not likely cause multigenic diseases including DN. Rather an accumulation of small changes in multiple genes more likely causes these diseases. Accordingly, even though individuals with NOS3 polymorphisms that are associated with mild reduction of NO production are associated with DN, whether the mild decrease in NO production from eNOS is sufficient to cause DN is not clear from the studies of homozygous eNOS -/- mice. To tackle this problem we took advantage of hybrid

	eNOS-/- db/db	eNOS-/- high-dose STZ	eNOS-/- low-dose STZ	eNOS-/- low-dose STZ, HF	eNOS-/- Akita	eNOS+/- Akita
	Ref.16	Ref.15	Ref.40	Ref.14	Ref.47	Ref.47
u-albumin	↑	↑	↑	↑	↑	↑
GFR	↓	not determined	↓	↓	↓	↑
GBM thickening	+	+	+	+	+	+
mesangial expansion	+	+	+	+	+	+
mesangiolysis	+	+	+	+	+	+
nodular sclerosis	+	+	+	+	+	+
tubulointerstitial fibrosis	-	+	-	+	-	-
arteriolar hyalinosis	+	+	not determined	-	-	-

Table 1: Features of diabetic nephropathy in mice with decreased eNOS expression.



all six genotypes is very informative. If heterozygotes are included, the functional consequences of different levels of expression of eNOS on diabetic complications can be determined. If WT and mutant eNOS mice are studied on both diabetic and non-diabetic backgrounds, additive, super-additive or sub-additive interactions between eNOS and diabetes can be detected. More generalized precautions can be found at DCC website: <<http://www.diacomp.org/shared/showFile.aspx?doctypeid=3&docid=79>>.

The expression of eNOS in glomeruli of the heterozygous eNOS ^{+/+} mice is approximately 30% of that of WT, which is comparable to NO production of TT genotype compared to that of GG genotype of NOS3 polymorphism G894T [47]. Using these mice it was shown that mild decrease in eNOS expression in eNOS ^{+/+} mice is sufficient to exacerbate DN. As demonstrated in eNOS ^{-/-} STZ mice, eNOS ^{+/+} Akita and eNOS ^{-/-} Akita mice develop DN likely due to activation of coagulation system [47].

High -dose STZ regimen

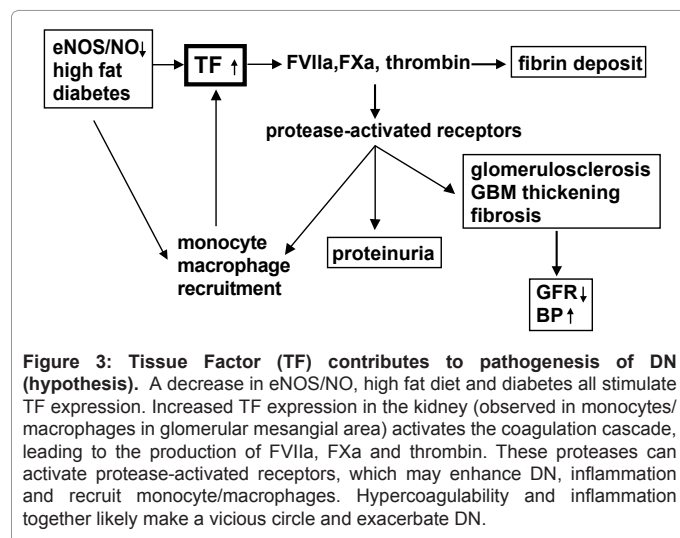
Nakagawa's group first reported diabetic renal injury using high dose STZ (100mg/kg, 2 days) in eNOS ^{-/-} mice [15]. The pathological changes in these mice also meet the criteria of DN proposed by DCC. The survival rate dramatically decreased in diabetic eNOS ^{-/-} mice compared to WT diabetic mice 5 months after inducing diabetes. These mice had higher BP than non-diabetic eNOS ^{-/-} mice, increased urinary albumin excretion and an elevation of BUN. The early features of DN in these mice include mesangial expansion and thickening of basement membrane. In addition, characteristics of advanced DN also developed after 5 months of diabetes, including mesangiolysis, capillary microaneurysms, nodular lesions and glomerulosclerosis. These authors suggested that uncoupling of VEGF with eNOS/NO can cause DN [48-50], because surprisingly high expression of VEGF was observed in diabetic eNOS ^{-/-} mice and because uncoupling causes excessive endothelial cell proliferation in response to VEGF *in vitro* [48]. They also found that diabetic eNOS ^{-/-} mice have macrophage infiltration, which is associated with severe glomerular injury. They suggest uncoupling of VEGF-eNOS/NO plays a role in macrophage migration [51].

eNOS ^{-/-} db/db mice

Zhao et al. [16] have introduced eNOS deficiency into *db/db* mice to study diabetic renal injuries in a model of type II diabetes. Although blood glucose levels of eNOS ^{-/-} *db/db* mice did not differ from those of *db/db* mice, lack of eNOS undoubtedly exaggerates DN including dramatic albuminuria, arteriolar hyalinosis, increased GBM thickness, mesangial expansion, mesangiolysis and focal segmental and early nodular glomerulosclerosis. In addition, eNOS ^{-/-} *db/db* mice exhibit decreases in GFR to levels < 50% of that in *db/db* mice.

Therapeutic approaches in eNOS ^{-/-} diabetic mice

Nakagawa's group and Harris's group have tested the effects of several drugs on DN of their eNOS ^{-/-} diabetic mice. Nakagawa's group has found that hydralazine, an antihypertensive drug, significantly lowered BP and ameliorated glomerular injuries in their eNOS ^{-/-} STZ-diabetic mice. But albuminuria and tubulointerstitial injury were not improved by hydralazine [52]. They also reported that spironolactone (a mineralocorticoid receptor antagonist) is superior to enalapril (an ACE inhibitor) or telmisartan (an angiotensin receptor blocker) in protecting eNOS ^{-/-} STZ diabetic mice, evidenced by reduction in BP,



vigor and genetic uniformity of the F1 progeny (eNOS ^{+/+}, ^{+/-}, or ^{-/-} with or without Akita diabetic mutation) of a cross between heterozygous 129SvEvTac eNOS ^{+/+} inbred females and C57BL/6J eNOS ^{+/+} inbred males carrying the dominant Akita diabetogenic mutation in *Ins2* gene. In order to carry out this type of experiment, it is extremely important to obtain mice of the critical genotypes at or close to the same time, so that one can measure parameters in a way that minimizes seasonal changes, dietary differences, any differences in the degrees of backcrossing, or unplanned differences in genotypes. Note that both parents, although inbred, are heterozygotes for the eNOS knockout, which are almost always healthier and have more offspring than homozygous mutants. The offspring are F1 hybrids (genetically as uniform as inbreeds but hardier). Note also that this mating produces the all of possible genotypes as littermates. Studying

urinary albumin excretion and renal injury [53]. Harris' group has treated eNOS $-/-$ db/db mice with captopril (ACEI) and triple drugs (hydralazine, reserpine, hydrochlorothiazide) at doses that reduce BP to the similar levels and found that captopril was more beneficial than triple therapy in ameliorating DN including urinary albumin excretion and glomerular injury. These data suggest that ACEIs have additional beneficial effects on eNOS $-/-$ db/db mice, which is beyond lowering BP. The authors suggest that there is unknown mechanism(s) underlying the renoprotective benefit of RAS blockade besides increasing eNOS/NO [54]. Different kinds of diabetic eNOS $-/-$ mouse models reviewed here provide strong evidence that eNOS/NO play a critical role in DN. Following this reasoning, NO donors or reagents that activate or prolong the second message of NO, such as cGMP could be useful for treatment of DN. Indeed, Kuno et al. [55] reported that sildenafil (a PDE5 inhibitor) attenuated DN in Otsuka Long-Evans Tokushima Fatty rats.

Conclusion

The diabetic eNOS $-/-$ mice are an excellent model of human DN. Although hypercoagulability is well known in diabetes, it has not been widely considered to cause DN. Because hypercoagulability caused by increased TF exacerbates DN, treatment against hypercoagulability might be useful for treating or preventing DN besides RAS blockade.

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