Published in final edited form as:

J Thromb Haemost. 2010 October ; 8(10): 2122–2132. doi:10.1111/j.1538-7836.2010.03976.x.

Elevated tissue factor expression contributes to exacerbated diabetic nephropathy in mice lacking eNOS fed a high fat diet

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Summary

Background—Human eNOS (*NOS3*) polymorphisms that lower its expression are associated with advanced diabetic nephropathy (DN), and the lack of eNOS accelerates DN in diabetic mice. Diabetes is associated with fibrin deposition. Lack of nitric oxide and fatty acids stimulate the NF-kB pathway, which increases tissue factor (TF).

Objectives—To test the hypothesis that TF contributes to the severity of DN in the diabetic eNOS-/- mice fed a high fat (HF) diet.

Methods—We made eNOS-/- and wild type mice diabetic with streptozotocin. Half of them were placed on a high fat (HF) diet.

Results—Blood glucose levels were not affected by either the diet or eNOS genotype. Lack of eNOS in the diabetic mice increased urinary albumin excretion, glomerulosclerosis, interstitial fibrosis, and glomerular basement membrane thickness. HF by itself did not affect DN in the wild

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Addendum: F Li, CH Wang, JG Wang, T Thai, G Boysen, L Xu, AL Turner, A Wolberg performed the experiments and analyzed the data. F Li, N Mackman, N Maeda co-wrote the manuscript. N Takahashi conceived the research, analyzed data, and co-wrote the manuscript.

Disclosures: None.

type mice, but significantly enhanced DN in eNOS-/- mice. More than half of diabetic eNOS-/- mice on HF died prematurely with signs of thrombotic complications. Diabetic kidneys contained fibrin and TF, and their levels were increased by the lack of eNOS and by HF in an additive fashion. The HF diet increased the kidney expression of inflammatory genes. The increase in TF preceded DN, and administration of an anti-mouse TF antibody to diabetic mice reduced the expression of inflammatory genes.

Conclusion—Together, these data indicate a causal link between TF and the exacerbation of DN in eNOS-/- mice. The condition is significantly worsened by enhanced inflammatory responses to a HF diet via TF.

Keywords

tissue factor; diabetic nephropathy; streptozotocin; coagulation

Introduction

Clinical studies have shown that 30-40% of diabetic patients develop DN [1], which is the most frequent cause of chronic kidney disease and is also a risk factor for stroke and heart attack [2]. The development and severity of DN vary greatly from one patient to another, with familial clustering suggesting that genetic factors play an important role [3]. Current knowledge of the susceptibility genes for DN and of their interactions with environmental factors is, however, very limited.

The mouse is a relatively poor model for DN, exhibiting only a limited extent of the pathology. Recently, however, multiple laboratories have demonstrated that diabetic mice that lack eNOS develop human like nephropathy [4-6]. These observations, together with the well-documented associations between advanced nephropathy in patients with either type I or type II diabetes and *NOS3* gene polymorphisms that lead to reduced expression of eNOS [7, 8], underscore the facilitating role of endothelial dysfunction in the pathogenesis of DN. In addition to a blunted acetylcholine-induced vasodilatation, mice lacking eNOS have high blood pressure (BP), insulin resistance, and glucose intolerance without obvious kidney abnormalities [9, 10]. Nitric oxide (NO) generally acts as an antioxidant and exerts anti-thrombotic effects [11]. Under some circumstances, possibly including diabetes, however, eNOS might actually increase oxidative stress [12]. Thus, the precise molecular pathways underlying the accelerated DN associated with eNOS deficiency warrant investigation.

In the present study, we tested the hypothesis that hypercoagulability is a key factor determining the severity of nephropathy in diabetic eNOS-/- mice. We focused our attention on tissue factor (TF), which initiates the main coagulation cascade. TF expression is induced by the activation of the NF-kB pathway [13, 14], which is inhibited by NO [15] and stimulated by fatty acids [16]. Accordingly, we investigated the effects on DN of the lack of eNOS and a high fat (HF) diet that is adjusted to diets consumed in Western societies. We here show that these two factors additively increase TF expression in diabetic kidneys, and that, when combined, they dramatically exacerbate DN. Administration of anti-TF antibody corrected the increase in the expression of inflammatory genes in the kidney of diabetic mice

by lack of eNOS and HF, indicating that TF contributes to the severity of DN in diabetic eNOS-/- mice fed HF.

Methods

Animals

Animal experiments were conducted in accordance with the guideline of IACUC at UNC at Chapel Hill. Male eNOS -/- mice (*Nos3*^{tm1Unc} [9]) and their WT littermates, backcrossed at least 10 times to C57BL/6J, were used in this study. Diabetes was induced in 4~6-month-old male mice by intraperitoneal injection of streptozotocin (STZ, 40 mg/kg, Sigma) for 5 consecutive days after 4-hour fasting as previously described [17]. Animals were maintained without insulin treatment for 6 months. Mice having plasma glucose concentration equal to or greater than 300 mg/dl throughout the study after STZ injection were defined as diabetic and included in the study. Both diabetic and non-diabetic control mice (injected with buffer only) were randomly divided and fed either normal chow (NC, 14% calories from fat) or a HF diet (42% calories from fat, TD88137, Harlan Teklad). At 3 and 6 months after inducing diabetes, the individual mice were placed in metabolic cages. Body weight, food and water intake, and urine volume were measured at 24 and 48 hrs. Blood samples were collected at the end of the metabolic cage study. Mice were then sacrificed for further analysis. For testing short-term effect of diabetes, 3~4 month-old male eNOS-/- and WT mice were injected with STZ or buffer only, fed with NC or HF, and analyzed 5 weeks later. To test whether TF exacerbates DN a single dose at 100 μg/mouse of an anti-mouse TF neutralizing antibody AF3178 (R&D) was administered intraperitoneally 5 weeks after STZ injection. Mice were sacrificed 4 days later for further analysis. [Our data show that 100 μg/mouse of anti-TF neutralizing antibody inhibits 70 % of the TF activity in the kidney without bleeding complication.]

BP and glomerular filtration rate (GFR) measurements

BP was measured by the computerized tail-cuff method for 6 days [18]. All mice were trained 10 cycles of measurements on the BP apparatus before 30 measurements were made each day. GFR was estimated by measuring the plasma and urinary creatinine using LC-MS/MS [19].

Biochemical measurements

Urinary albumin was determined using Albuwell-M kits (Exocell Inc.). Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) was measured with an ELISA kit (The Japan Institute for The Control of Aging). Plasma CML was measured with an ELISA kit (CycLex Co.). Plasma thrombin-antithrombin (TAT) complexes were measured with the AssayMax TAT complexes ELISA kit (Assaypro Co.). For PT and aPTT determination, blood was collected in glass tubes containing 3.8% trisodium citrate (1 vol citrate plus 9 vol blood), and PT and aPTT were measured using a thromboplastin reagent and aPTT reagent (Biomerieux Inc.), respectively [20, 21]. Kidney cortex (10 mg) was homogenized in 1ml PBS for measuring CML content [22] and TF-dependent procoagulant activity [23].

Quantitative RT-PCR

The kidney tissue was snap frozen in liquid nitrogen, and the RNA was extracted using Trizol (Life Technologies). Gene expression was quantified with TaqMan real-time quantitative RT-PCR (Applied Biosystems) with β-actin as a reference gene [18]. The primers and probes used are listed in the Online Appendix Table 1.

Kidney morphometry and immunohistochemistry

Cross paraffin sections of kidneys containing papilla (4 μm thick) were stained with Periodic Acid-Schiff (PAS) and with Masson's trichrome, and scanned using a NIKON Microphot-FCA 216567. Glomerulosclerosis was defined as synechiae formation with global obliteration of the capillary loops [24]. The number of sclerosed glomeruli was expressed as a percentage of the total number of glomeruli. The mesangial matrix score was defined as the ratio of the mesangial matrix area divided by the glomerular tuft area [24, 25], and was measured using the Image J program. All of the glomeruli (90 to 110) in each section were measured. Tubulointerstitial fibrosis was scored by using 10~15 fields of the cortex at a magnification of × 4 with a scale of 0 to 4: 0, no fibrosis; 1, <25%; 2, 25-50%; 3, 50-75%; 4, >75% of the field with interstitial fibrosis [25]. For GBM thickness, transmission electron micrographs at a magnification of ×5,000 were printed, and the thickness of the GBM of capillary loops was measured manually. Rabbit anti-human fibrinogen polyclonal antibody (1:2000; Dako A0080) was used for immunohistochemical detection of fibrin. This antibody recognizes mouse fibrin but not mouse fibrinogen [26, 27]. Monoclonal rat anti-mouse TF antibody (1H1) was kindly provided by Dr. Daniel Kirchhofer (Genetech) [28]. The antigen was retrieved with a citrate buffer method [29]. For double staining of TF and MOMA-2, a monoclonal rat antibody against mouse MOMA-2 (1:25, AbD Serotec) preincubated with goat anti-rat immunoglobulins labeled with FITC (1:50, Jackson ImmunoResearch), followed by 1H1 preincubated with goat anti-rat immunoglobulins labeled with Rhodamine Red-X (1:50, Jackson ImmunoResearch) were used. Sections were analyzed using confocal laser scanning microscopy LSM5PASCAL (Carl Zeiss).

Mouse mesangial cell culture

Mouse mesangial cells (ATCC, CRL-1927) were cultured in DMEM with high glucose (4g/L) containing 10% (vol/vol) FBS, 10 mg/ml streptomycin, and 100 U/ml penicillin at 37°C in 5% CO_2-air. Two days after confluence, FBS was replaced by 0.5% mouse serum for 48 hrs. Cells were then treated with mouse factor VIIa (50 nM, generous gift from Dr. Lars Petersen, Novo Nordisk, Bagsvaerd, Denmark) for 6 hrs, and harvested and SNAP frozen in liquid nitrogen for further analysis.

Statistical analysis

Data were expressed as mean ± SEM. Multifactorial ANOVA with the program JMP 8.0 (SAS Institute Inc.) was used to compare the effects of diabetes, eNOS genotypes, diets, and of their combinations. Tukey-Kramer HSD test was used for posthoc comparisons between each group.

Results

Survival of diabetic mice

The eNOS-/- mice and their WT littermates were made diabetic by repeated injections of low-dose streptozotocin (STZ) at 4-6 months of age. Control non-diabetic groups were treated with buffer only. Both the diabetic and non-diabetic mice were randomly divided into two groups and fed either normal chow (NC) or a high fat diet (HF) for 6 months. The general characteristics of the diabetic mice are presented in Online Appendix Table 2. Because there were no remarkable differences in the DN parameters measured among the four non-diabetic groups (WT NC, WT HF, eNOS-/- NC, eNOS-/- HF), we focus only on the data from the four groups of diabetic mice in the following analysis, unless otherwise noted.

The absence of eNOS increased tail-cuff BP by approximately 15~20 mmHg (p<0.0001), but did not affect the plasma glucose levels. BP was not affected by diabetes or by the diet, and did not significantly change in the 6 month study in any group of mice. eNOS deficiency caused premature death in diabetic mice, and 4 out of 11 (~30%) of the diabetic eNOS-/- mice fed NC died by 6 months after the induction of diabetes (Fig. 1A). Although HF did not affect the survival of diabetic WT mice, 8 out of 14 (~60 %) of the diabetic eNOS-/- mice fed HF died (Fig. 1A). In some cases at necropsy there was evidence of clots in the mesenteric arteries and necrosis of the small intestine, suggesting ischemic events. Some exhibited massive bleeding in the abdominal cavity or in the lung (Online Appendix Fig. 1) without clear evidence of dissection of the aorta, stroke, myocardial infarction, or apparent intravascular coagulations in other tissues, including the liver, heart and brain (not shown). We conclude that lack of eNOS causes premature death of diabetic mice involving hypercoagulability, and that HF accelerates premature death only when the animals are deficient of eNOS.

Diabetic nephropathy (DN)—When the animals were fed NC, eNOS-/- mice that were diabetic for 6 months had increased urinary albumin excretion without significant decrease in creatinine clearance compared to diabetic mice with WT eNOS (Figs. 1B, C). Diabetic eNOS-/- mice fed NC had a severe glomerulosclerosis that was not seen in any of the diabetic WT mice (Figs. 2B, 2E). They also had severely exacerbated tubulointerstitial fibrosis and thickening of glomerular basement membrane (GBM) (Figs. 2G, 2H).

The high fat diet did not alter kidney function in diabetic WT mice, although in eNOS-/- mice albuminuria and GFR showed a trend of worsening by HF (Figs. 1B,1 C). HF did not significantly increase the mesangial score and the tubulointerstitial fibrosis score compared to NC regardless of eNOS genotype (Figs. 2F, 2G), but more than doubled the number of glomeruli with sclerosis in the diabetic eNOS-/- mice (Figs. 2D-2E, online Appendix Fig. 2). HF also significantly increased GBM thickness in the diabetic eNOS-/- mice (Fig. 2H). In transmission electron micrographs, endothelial cells, fenestrae, and podocytes appeared normal in the kidneys of all mice, while thickening of GBM in eNOS-/- diabetic mice on NC and HF was evident (online Appendix Fig. 3). We conclude that the lack of eNOS accelerates both the functional and morphological changes that accompany DN, but that HF accelerates it only when the animals are deficient of eNOS.

Kidney TF and inflammation—To investigate if there was an activation of coagulation in DN, fibrin deposition in the kidneys was examined. There was no apparent fibrin deposition in the kidneys of non-diabetic mice regardless of the diet or eNOS genotype (not shown). However, the glomeruli of all of the diabetic mice were positive for fibrin (Fig. 3A). Diabetic eNOS-/- mice fed NC showed increased fibrin deposits in the glomeruli compared to WT control mice (Fig. 3A top panels). HF also increased fibrin deposition in the glomeruli, and the diabetic eNOS-/- mice on HF had the most abundant fibrin deposits in their glomeruli among all groups (Fig. 3A bottom panels).

Because TF initiates blood coagulation, we measured TF expression in the kidney and the TF-dependent clotting time of kidney homogenates as an index of kidney TF activity [23]. Both eNOS deficiency and HF significantly and additively increased renal TF mRNA levels and activities (Figs. 3B, 3C). Again, diabetic eNOS-/- mice on HF had the highest TF expression and activities. The glomeruli of non-diabetic mice were negative for immunoreactive TF (not shown). However, glomeruli of all diabetic mice were positive for TF immunoreactivity, and diabetic eNOS-/- mice fed HF showed the most prominent TF immunoreactivity in their glomerular mesangial area (Fig. 3D). All cells in the kidney that were strongly positive for TF were monocytes/macrophages, while no MOMA-2 positive cells were negative for TF (Fig. 3D). No apparent TF immunoreactivity was detected outside glomeruli. We conclude that both lack of eNOS and HF independently and additively increase kidney TF, HF increases kidney TF regardless of the presence or absence of eNOS, and that TF was co-localized with monocytes/macrophages in the glomeruli.

TF, a lack of eNOS and diabetes are all known to enhance inflammation [30-32]. Thus, we next quantified the gene expression in the kidney of MCP-1, ICAM-1, VCAM1, IL-1β, TNFα, and IL-6. Diabetes markedly increased the expression of all of these genes (Online Appendix Table 3). In non-diabetic mice HF significantly increased the expression of ICAM-1 and VCAM-1 by 50% to 200%, while the lack of eNOS itself had no significant effect. However, when mice were diabetic, the lack of eNOS significantly increased the renal expression of ICAM-1 and VCAM-1. Lack of eNOS and HF synergistically increased the expression of IL-1β and IL-6 in diabetic mice. Furthermore, linear regression analyses at the individual animal level showed that the levels of urinary albumin excretion, GBM thickness, and tubulointerstitial fibrosis were all strongly correlated with TF levels (Online Appendix Fig. 4). These data demonstrate that diabetes and lack of eNOS interactively increase the expression of cell adhesion molecules in the kidney. The data also strongly suggest that a HF diet contributes to the exacerbation of DN in diabetic eNOS-/- mice through the stimulation of inflammatory processes.

In order to test whether the coagulation abnormalities are systemic or are restricted to the kidney, we measured the prothrombin time, PT, and activated partial thromboplastin time, aPTT. The lack of eNOS and HF themselves had no effect on these two parameters (Table 1). However the diabetic eNOS-/- mice on HF had significantly shorter aPTT than other groups of diabetic mice, although reduction of their PT did not reach significance. A lack of eNOS and HF in diabetic mice tended to increase circulating thrombin-anti-thrombin (TAT) complexes, a sensitive marker of thrombin formation. When combined, the lack of eNOS and HF caused an increase in circulating TAT complexes. These results demonstrate that the

absence of eNOS increases systemic hypercoagulability in diabetic mice, and the condition is significantly exacerbated further by HF.

Role of TF in the exacerbation of DN in mice lacking eNOS—We now know that TF is associated with DN, but whether an increase in TF is a cause or the result of DN is not clear. If TF contributes to DN, an increase in TF precedes the development of DN. If TF is a result of DN, TF does not increase until DN develops. To test this, we examined eNOS-/- and WT mice 5 weeks after diabetes were induced by STZ. At this early time points, urinary albumin excretion did not significantly increase (Fig. 4A). There was no difference in urinary albumin excretion and GFR between diabetic and non-diabetic mice in both WT and eNOS-/- mice even when the animals were 3 months of diabetes except eNOS-/- diabetic mice on HF had higher urinary albumin excretion than non-diabetic eNOS-/- mice on HF (online Appendix Figure 5). Kidneys from both eNOS-/- and WT mice that were diabetic for 5 weeks did not show apparent histological changes suggestive of DN (Fig. 4B). However, the renal expression of TF in the diabetic mice was already significantly higher than that of non-diabetic mice, reaching to the similar levels as those at 6 months of diabetes (online Appendix Fig. 6). Diabetic eNOS-/- mice had higher TF expression than diabetic WT mice (Fig. 4C). Similar to kidneys in mice that were diabetic for 6 months, TF is expressed in macrophages in glomeruli at this early stage of diabetes (Fig. 4D). All cells in the kidney that were strongly positive for TF were monocytes/macrophages; no MOMA-2 positive cells were negative for TF. No TF immunoreactivity was detected outside glomeruli.

Although TF is mainly expressed in macrophages residing in mesangial area but not in mesangial cells *in* vivo, it could still activate coagulation cascade in mesangial area using coagulation factors from the circulation, and facilitate inflammatory and fibrogenic gene expression. Consistent with this possibility, treatment of mouse mesangial cells CRL-1927 with mouse Factor VIIa, in culture significantly increased the expression of MCP-1, but not TGFβ1 or type IV collagen in 6 hrs (Online Appendix Fig. 7).

We next tested whether blocking TF can abrogate the exacerbation of DN *in vivo.* Five weeks after induction of diabetes and fed either NC or HF, we administered to half of the animals a polyclonal anti-mouse TF neutralizing antibody AF3178 (100 μg/mouse *i.p.* once), and to the other half normal goat IgG. Four days later animals were sacrificed and kidneys analyzed for TF activities and inflammatory and fibrogenic gene expression. The anti-TF antibody decreased TF activity in the kidneys by 70%, and significantly suppressed the renal expression of several inflammatory and fibrogenic genes in diabetic WT mice fed either NC or HF (Fig. 5). Remarkably the anti-TF antibody reduced the induction of inflammatory and fibrogenic genes in the kidneys from diabetic eNOS-/-mice fed NC (Fig. 5). Unfortunately, we do not have data from the eNOS-/- mice fed HF because most of them died before administering anti-TF neutralizing antibody. Taken together, these data indicate that TF increases earlier than the development of DN, enhances inflammation in mesangial area, and plays a critical role in the pathogenesis of DN induced by a lack of eNOS and HF diet.

Influence of BP and of oxidative stress on DN in mice lacking eNOS

High BP is a factor known to accelerate DN [30]. The BP values of the eNOS-/- mice were higher than the WT mice by approximately 15~20 mmHg (Online Appendix Table 2). However, linear regression analysis showed that urinary albumin excretion of the individual animals is only very weakly correlated with BP ($p=0.06$, $R^2=0.16$), and accounted for less than 20% of the variance in the parameters of DN, and thus renal injury in diabetic eNOS-/- mice is largely independent of BP. While the lack of eNOS may lead to ischemia from intense vasoconstriction in the kidney and renal injury, expression of the HIF-1α gene was similar among all groups of mice (Online Appendix Fig. 8), suggesting that ischemia is not a likely cause of renal injury in our mice.

Oxidative stress also plays an important role in DN [31], and diabetes significantly increases the markers of oxidative stress such as plasma and renal N^{ε}-carboxymethyllysine (CML) and urinary 8-hydroxy-2′-deoxyguanosine (8OHdG) (not shown). Neither the lack of eNOS nor a HF diet increased the levels of these markers in diabetic mice by itself (Online Appendix Fig. 9). Surprisingly, however, lack of eNOS and HF synergistically decreased plasma CML and urinary 8OHdG. These data indicate that the exacerbation of DN caused by lack of eNOS and HF is not due to an increase in oxidative stress.

Discussion

We have demonstrated that the lack of eNOS causes premature death of diabetic mice and exacerbates DN with signs of thrombotic complications. A high fat (HF) diet by itself did not cause premature death or exacerbation of DN of diabetic WT mice, but in the absence of eNOS HF further decreased survival rate and accelerated DN. Diabetic eNOS-/- mice fed a HF diet show more severe diabetic glomerulosclerosis than that reported to date in any experimental animal model. When the coagulation pathway is highly activated, large amounts of coagulation factors are produced and consumed, which can lead to clotting, necrosis and bleeding. Although we do not know the values of platelets and coagulability in the animals that died with bleeding, consumption coagulopathy is a likely explanation of the hemorrhagic events we observed in some of the eNOS-/- diabetic mice.

The role of NO in thrombus formation is controversial [11, 32]. Kidneys from our non-diabetic mice, regardless of their eNOS genotype and diet, did not exhibit fibrin deposition, indicating that the absence of eNOS alone is not sufficient to induce the deposition of fibrin. In contrast, all diabetic mice showed fibrin deposition in the kidneys, and both the lack of eNOS and HF markedly increased renal fibrin deposition only when the animals were diabetic (Fig. 3A). Fibrin deposition in the glomeruli has also been reported in IgA nephropathy and mesangioproliferative glomerulonephritis [33, 34]. In these conditions, coagulation in the glomeruli leads to extracellular matrix accumulation [33]. The mesangial expansion and glomerulosclerosis in the kidneys of our diabetic animals may also be the sequelae of fibrin deposition in the glomeruli.

TF triggers coagulation cascade. NO inhibits [15] and HF stimulates the NF-kB pathway [35], one of the most important regulators of TF expression [13, 14]. Accordingly, we investigated the effects of eNOS and HF on TF. The results obtained here show that lack of

eNOS and HF independently and additively increased TF expression in monocytes/macrophages of the kidney glomeruli (Figs. 3B, 3C, 3D). These results are consistent with previous observations that TF expression is increased in monocytes from diabetic patients [36, 37].

Lack of eNOS increased TF expression and activity, and had significant effects on parameters of DN. In contrast, HF increased TF expression and activity in diabetic WT mice to a similar degree as lack of eNOS did, but it did not significantly exacerbate DN. However, HF had significant effects in glomerulosclerosis and GBM thickness in diabetic eNOS-/- kidneys. It is likely that eNOS-derived NO protects kidneys from TF-induced damage, and that lack of eNOS discloses the TF-induced renal damage. HF causes numerous abnormalities including inflammation, obesity, hypertension, and insulin resistance. However, anti-TF antibody reduced the increase in expression of inflammatory and fibrogenic genes in diabetic mice fed HF, suggesting the pivotal role of TF in HF-induced inflammation. This supports our conclusion that increased TF expression, which precedes overt DN, plays an important role in the pathogenesis of DN in diabetic eNOS-/- mice.

Increased TF expression in the kidney activates the coagulation cascade, leading to the production of factor VIIa, factor Xa and thrombin. These proteases can activate protease-activated receptors, which may enhance DN [38]. TF can also mediate inflammation [39]. Indeed lack of eNOS and HF synergistically increased inflammatory gene expression in the kidneys of diabetic mice (on-line Appendix Table 3). The expression levels of these genes were found to be associated with the variations in DN parameters observed in the present study. Inflammation, in turn, promotes coagulation by further recruiting monocytes/macrophages, and by increasing TF expression, and exacerbates DN.

Although increased TF is associated with the severity of DN, it was not clear whether an increase in TF is a result or cause of DN. Our data from mice 5 weeks after induction of diabetes show that an increase in TF and inflammatory and fibrogenic gene expression precedes the development of DN. Moreover, an anti-TF neutralizing antibody reduced the increase in the expression of inflammatory and fibrogenic genes (Fig. 5). These data are consistent with the fact that TF can mediate inflammation [39], and strongly suggest that the increase in TF plays a causative role in the exacerbation of DN. TF expression in macrophages of the kidney glomeruli at both 5 weeks and 6 months of diabetes (Figs. 3D, 4D) is consistent with previous observations that TF expression is increased in monocytes from diabetic patients [36, 37]. Although macrophages likely infiltrate in mesangial area to clean up the aftermath of glomerular injury, macrophage TF likely causes further renal injury. 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 [40, 41]. When macrophages in mesangial area express TF and activate coagulation cascade, Factor VIIa, Factor Xa, and thrombin are produced, which could increase the expression of inflammatory genes such as MCP-1 in mesangial cells *in vitro* possibly through binding to protease activated receptors. Our data showing that FVIIa increases MCP-1 expression in mesangial cells *in vitro* are consistent with this hypothesis (online Appendix Fig. 7). These results suggest that the increase in TF likely causes the exacerbation of DN. Although they do not exclude the possibility that hypercoagulability is induced secondary to inflammation,

hypercoagulability and inflammation together likely make a vicious circle in exacerbating DN.

DN in eNOS-/- mice includes marked tubulointerstitial fibrosis, which requires some comments. Although all the eNOS-/- diabetic mice reported to date including this study show glomerulosclerosis [4-6], the development of tubulointerstitial fibrosis in these mice has been controversial [5, 42, 43]. For example, Kosugi et al. used high dose STZ to make the eNOS-/- mice diabetic, and observed robust tubulointerstitial damage [42]. In contrast, Kanetsuna et al. used low dose STZ and reported that diabetic eNOS-/- mice did not develop significant tubulointerstitial damage [6]. We used a similar low dose STZ, and observed that the lack of eNOS was sufficient to cause tubulointerstitial fibrosis. All of these three studies used eNOS-/- mice on C57BL/6J genetic background. Although tubulointerstitial damage of high dose STZ mice was reversed by administration of insulin, suggesting this phenotype is due to diabetes rather than to toxicity of STZ [42], high dose STZ eNOS-/- mice have about 30 mmHg higher tail-cuff BP than ours. This malignant hypertension could be triggered by STZ-induced tissue damage [43], and contribute to the development of tubulointerstitial fibrosis. Our observation that a HF diet enhances DN dramatically only in diabetic eNOS-/- kidneys suggests that other environmental factors that increase inflammatory burdens cannot be ignored.”

High BP is known to exacerbate DN [30], and it is likely that high BP plays some role in exacerbating DN of mice lacking eNOS as previously suggested [42]. However, as judged by linear regression analysis, the contribution of BP to DN in diabetic eNOS-/- mice is small in our experiment. In addition, although oxidative stress greatly contributes to the development of DN [44], our diabetic eNOS-/- mice showed reduced oxidative stress compared to the diabetic mice with wild type eNOS. This finding is consistent with the concept of eNOS uncoupling in diabetes, where eNOS generates superoxide rather than NO [45], and indicate that the protective role of eNOS in DN is not mediated by a reduction of oxidative stress caused by diabetes, but through prevention of monocytes recruitment to the mesangial area.

In conclusion, our data demonstrate that lack of eNOS and HF additively increase kidney TF expression in the diabetic kidneys, which likely causes acceleration of DN. HF stimulates inflammatory response in diabetic eNOS-/- mice without any further increase in oxidative stress compared to WT diabetic mice. The increase in BP caused by lack of eNOS appears to play a minimal role. These findings suggest that the major determinant of DN in eNOS-/- diabetic mice is an upregulation of coagulation and inflammation via increased TF expression. Taken together, it is suggested that hypercoagulability is more important in the pathogenesis of DN than has previously been recognized, and that kidney TF expression is an excellent marker of the severity of DN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Authors thank Dr. Daniel Kirchhofer (Genetech) for kindly providing the monoclonal rat anti-mouse TF antibody (1H1), Dr. Lars Petersen (Novo Nordisk, Bagsvaerd, Denmark) for generous gift of the murine factor VIIa, Mr. Leonard Collins for LC-MS/MS, and Drs. Joseph Galanko, Jonathon W. Homeister, J. Charles Jennette, and Oliver Smithies for advice and discussions. This work was supported by grants from American Heart Association (0265464U, 0855335E), the National Institutes of Health (U01 DK076131, P30DK56350, P30 ES10126).

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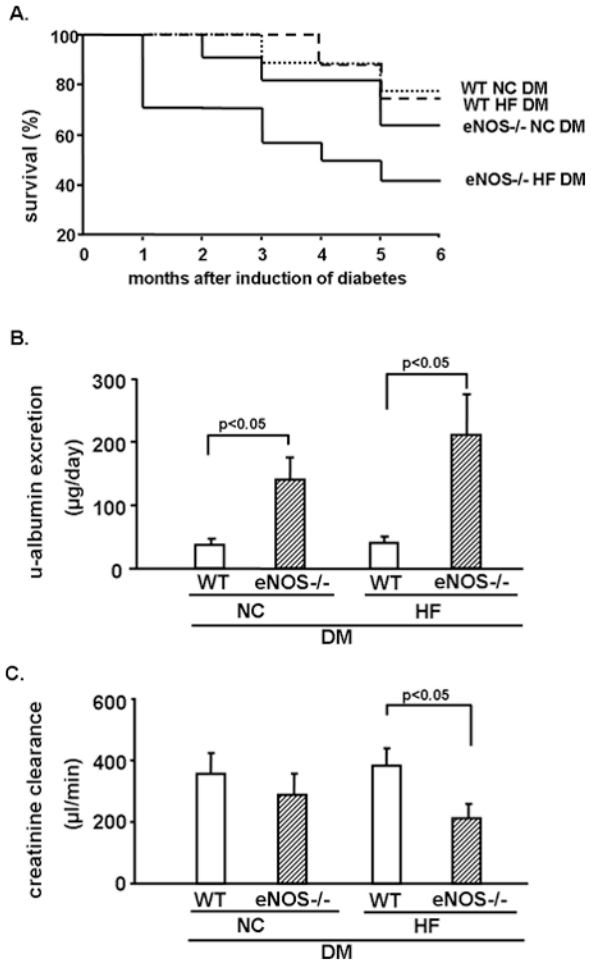

Figure 1. Survival rates and renal function after 6 months of diabetes

A, Survival rates of wild type (WT) and eNOS-/- diabetic mice fed normal chow (NC) or a high fat (HF) diet. B, daily urinary albumin excretion of diabetic mice. C, creatinine clearance of diabetic mice. Data are expressed as mean ± SEM, n≥6. The creatinine clearance of non-diabetic age-matched mice was: 275 ± 80 µl/min (WT on NC), 249 ± 77 (eNOS-/- on NC), 340 ± 65 (WT on HF), 322 ± 72 (eNOS-/- on HF).

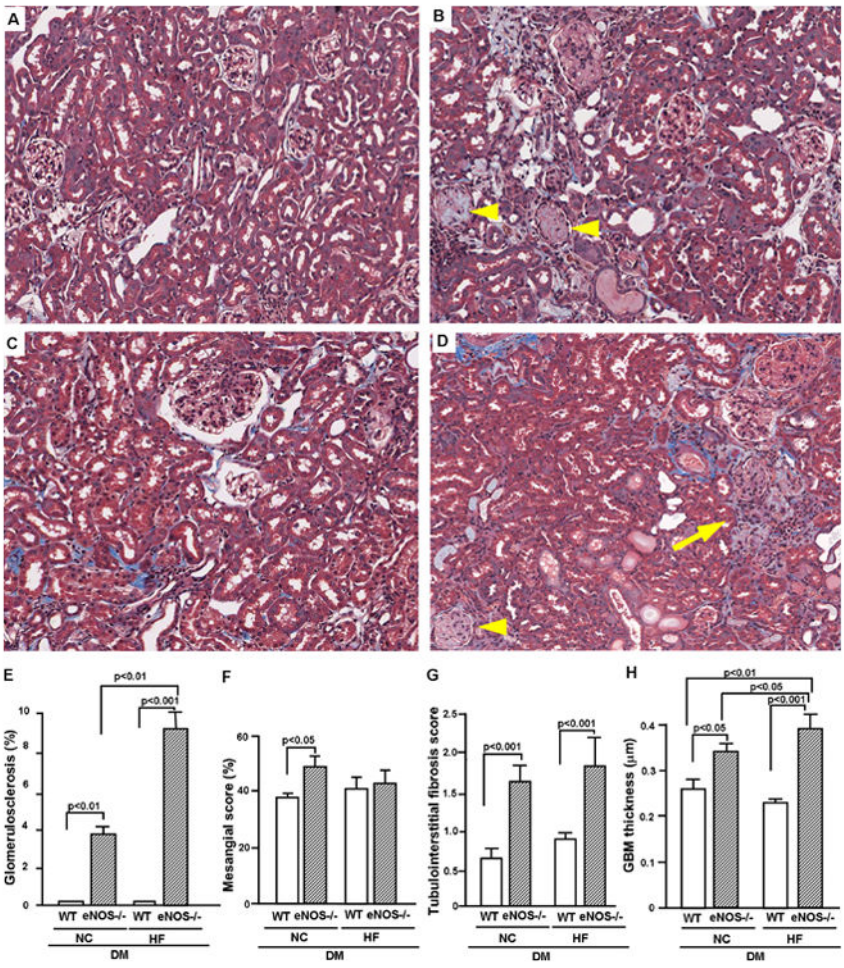

Figure 2. Renal morphology of the diabetic mice

A, wild type diabetic mouse fed normal chow; B, eNOS-/- diabetic mouse fed normal chow; C, wild type diabetic mouse fed a high fat diet; D, eNOS-/- diabetic mouse fed a high fat diet; E-H, quantification of histological changes. A-D, Masson Trichrome staining, original magnification × 40. The arrow heads and a yellow arrow show glomerulosclerosis and tubulointerstitial fibrosis, respectively. Higher magnification of glomeruli and transmission electron micrographs of glomerular capillary walls are shown in online Appendix Figs. 2 and 3.

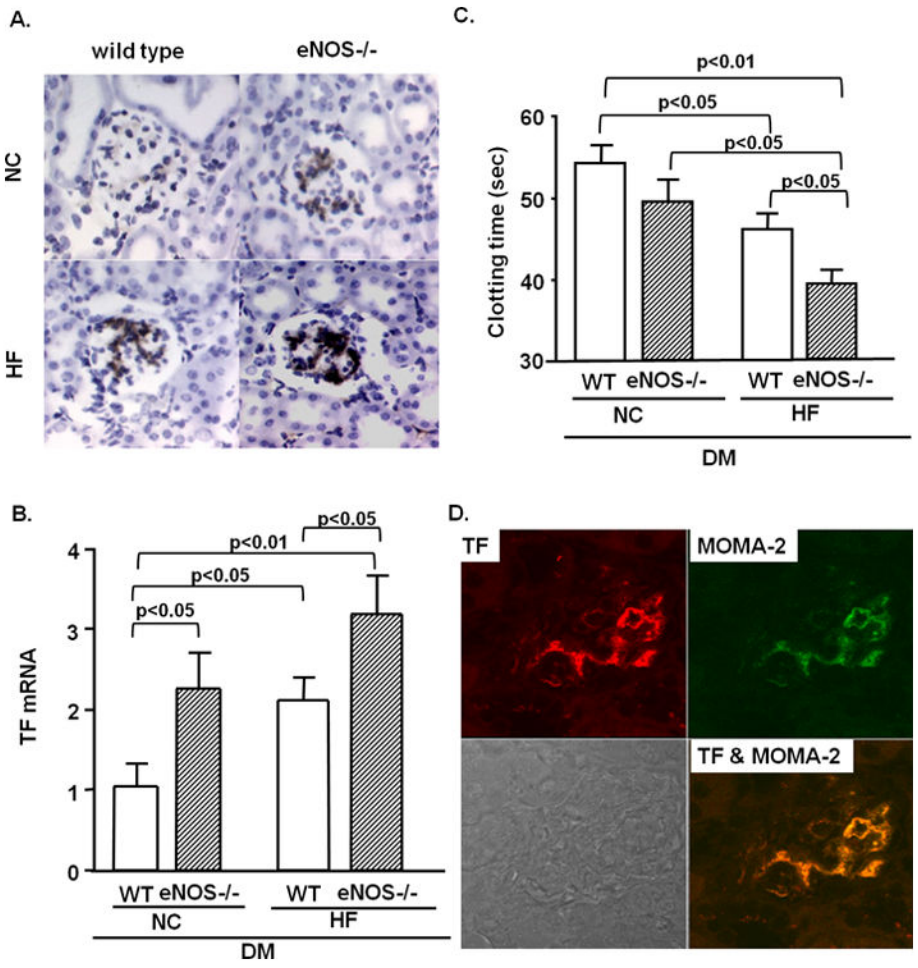

Figure 3. Fibrin deposit and Tissue factor (TF) expression in the kidneys of mice diabetic for 6 months

A. fibrin deposit in the kidneys of diabetic mice. Diabetic eNOS-/- mice on a high fat diet had prominent fibrin deposits in glomeruli. Original magnification ×100. B. kidney TF mRNA levels expressed in fold change relative to WT diabetic mice on NC. TF expression of 8 groups of mice including 4 non-diabetic groups can be found in online Appendix Fig. 6. $p<0.001$ for eNOS genotype effect, and diet effect. There was no interaction between eNOS genotype and diet. C, kidney cortex TF activities of diabetic mice. D, immunostaining against TF and monocytes/macrophages (MOMA-2) in a diabetic eNOS-/- mouse on a high fat diet. The left lower panel is a differential interference contrast image of the glomerulus.

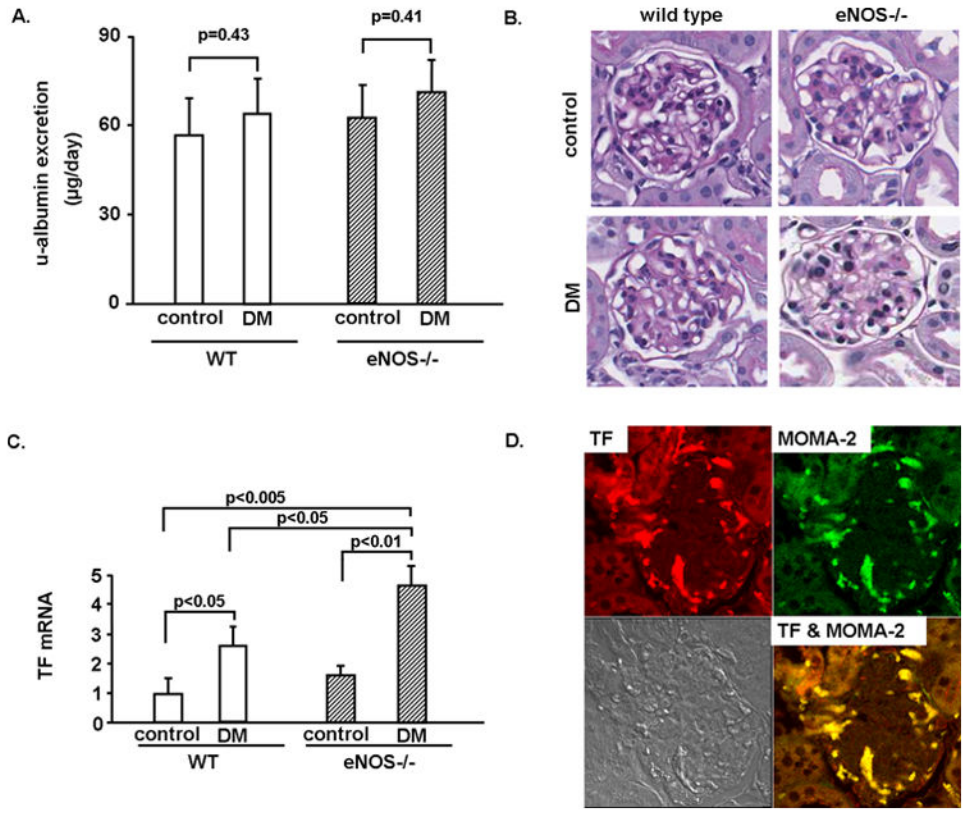

Figure 4. An increase in kidney tissue factor expression precedes the development of DN

Mice were diabetic for 5 weeks and fed normal chow. Urinary albumin excretion (A) and kidney histology (B. PAS staining, original magnification ×200) did not differ among the 4 groups. In contrast, TF mRNA expression (fold WT control) in the kidneys (C) was significantly increased by diabetes and by lack of eNOS. D, Immunostaining of glomeruli against TF and monocytes/macrophages (MOMA-2) in a diabetic eNOS-/- mouse. Data are expressed as mean ± SEM, n≥5 each group.

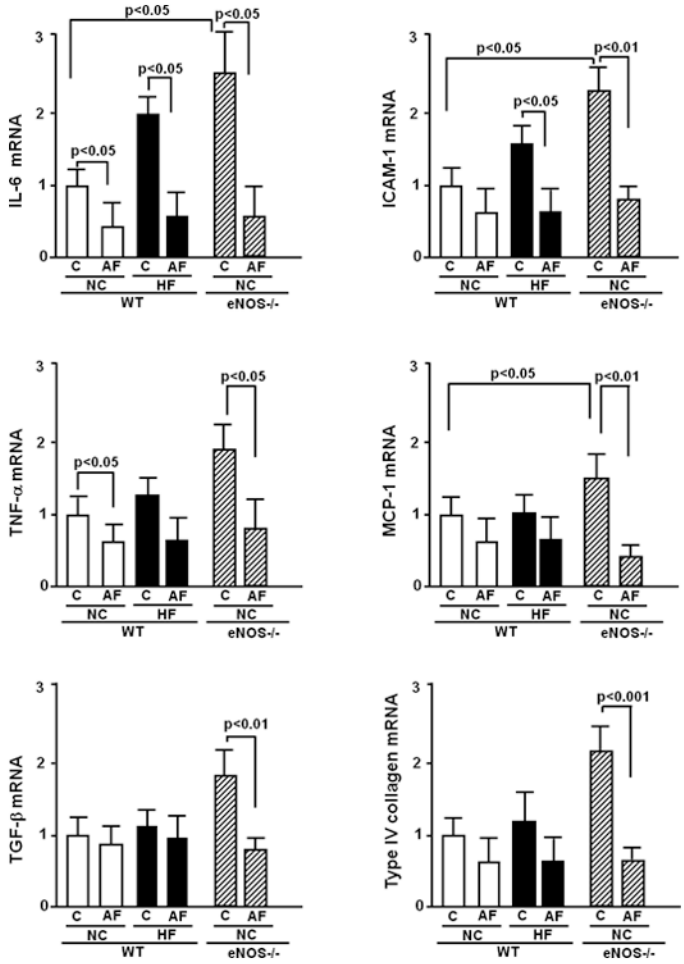

Figure 5. Attenuation of the expression of inflammatory and fibrogenic genes by an anti-mouse TF antibody AF3178

All mice were made diabetic with STZ. A single dose of AF3178 (100μg) was administered i.p. 5 weeks after STZ injection, and mice were sacrificed for analysis 4 days later. C: control, AF: AF3178, NC: normal chow, HF: high fat diet. Data are mean ± SEM relative to the mean of diabetic WT mice fed NC, n≥5.

Table 1

Plasma parameters of coagulation and inflammation in diabetic mice

genotype	diet	PLT (×10^3/μl)	PT (sec)	aPTT (sec)	TAT (ng/ml)	IL-6 (pg/dl)
WT	NC	243 ± 48	16.5 ± 2.3	43.6 ± 9.6	1.4 ± 0.2	36.6 ± 2.1
eNOS-/-	NC	436 ± 70	16.8 ± 2.7	43.8 ± 8.3	5.4 ± 1.2	37.4 ± 3.2
WT	HF	313 ± 103	14.9 ± 1.2	41.6 ± 6.5	3.7 ± 1.2	46.3 ± 6.0
eNOS-/-	HF	475 ± 123	11.0 ± 3.8	28.0 ± 5.8^{abc}	14.3 ± 3.9^{ac}	69.2 ± 3.1^{abc}

PLT: platelet counts, PT: prothrombin time, aPTT: activated partial thromboplastin time, TAT: thrombin anti-thrombin complex. WT: wild type, NC: normal chow, HF: high fat diet. Data are expressed as mean ± SEM (N≥5). a, b, c are significantly different from WT on NC, eNOS-/- on NC, and WT on HF, respectively.