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ORIGINAL ARTICLE

Is a reduced estimated glomerular filtration rate a risk factor for stroke in patients with type 2 diabetes?

Ryotaro Bouchi, Tetsuya Babazono, Izumi Nyumura, Kiwako Toya, Toshihide Hayashi, Mari Ohta, Ko Hanai, Yuka Kiuchi, Kumi Suzuki and Yasuhiko Iwamoto

Although chronic kidney disease is a risk factor for cardiovascular disease it is unclear whether diabetic patients with a reduced glomerular filtration rate (GFR), independent of (micro)albuminuria, carry an increased risk of stroke. We therefore investigated the independent effect of estimated GFR (eGFR) on stroke events in patients with type 2 diabetes mellitus (T2DM). We studied T2DM patients with an eGFR $\geqslant$15 ml min^{-1} per 1.73 m^2, who had no history of stroke. Patients were divided into four categories by the eGFR at baseline for comparison: $\geqslant$90, 60–89, 30–59 and 15–29 ml min^{-1} per 1.73 m^2. The end point was an incident stroke event. The Cox proportional hazard model was used to calculate the hazard ratio (HR) and 95% confidence interval (CI). The study included a total of 1300 T2DM patients (546 women and 754 men) with a mean ($\pm$s.d.) age of 63 $\pm$ 13 years. During a mean follow-up period of 3.7 $\pm$ 1.4 years, 91 patients experienced an incident stroke event. Although a lower eGFR was associated with an increased stroke risk using a univariate model, statistical significance disappeared after adjusting for other risk factors including albuminuria. The HR (95% CI) was 0.75 (0.40–1.41, *P*=0.373), 0.99 (0.50–1.95, *P*=0.964) and 0.91 (0.36–2.28, *P*=0.844) for patients with eGFRs of 60–89, 30–59 and 15–29 ml min^{-1} per 1.73 m^2, respectively, compared with patients with an eGFR $\geqslant$90. Clinical albuminuria remained a significant risk factor for stroke, and the adjusted HR compared with normoalbuminuria was 2.40 (1.46–3.95, *P*=0.001). In conclusion, the association between reduced GFR and stroke events in patients with T2DM is likely to be mediated by albuminuria.
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Keywords: albuminuria; diabetes; estimated glomerular filtration rate; stroke

INTRODUCTION

Chronic kidney disease (CKD)[1] has been recognized as an independent risk factor for cardiovascular disease mortality and morbidity in both Japanese[2] and Western populations.[3,4] The incidence of coronary heart disease and stroke differs between Western and Asian populations;[5] in Japan the stroke mortality is three-fold higher than in the United States, whereas coronary heart disease mortality is approximately one-third that in the United States.[6] The role of CKD in the pathogenesis and progression of stroke may therefore differ by ethnicity.

Diabetes is a strong risk factor for coronary heart disease as well as stroke. The hazard ratio (HR) of stroke in patients with type 2 diabetes mellitus (T2DM) is reported to be two- to five-fold that in the non-diabetic population.[7,8] In addition, the progression of diabetic nephropathy is associated with a higher incidence of cardiovascular diseases. A decreased estimated glomerular filtration rate (eGFR) has also been shown to be a new and independent risk factor for cardiovascular events and all-cause mortality in patients with T2DM;[9] however, evidence is lacking regarding whether or to what extent reduced eGFRs increase the risk of incident stroke events in diabetic patients. We therefore examined whether eGFR could predict the risk of stroke, independent of conventional cardiovascular risk factors and albuminuria in T2DM patients.

METHODS

Study population

This was a prospective hospital-based observational cohort study that included consecutive patients with T2DM who were admitted to the Department of Medicine, Diabetes Center, Tokyo Women's Medical University Hospital in Tokyo, Japan, from 1 January 2002 to 31 December 2003, for the purpose of glycemic control and evaluation of diabetic complications. Patients were eligible if they were 20 years old or older, had no history of stroke and had an eGFR $\geqslant$15 ml min^{-1} per 1.73 m^2. Patients undergoing renal replacement therapy, pregnant women and patients with infectious and/or malignant diseases were excluded. The diagnosis of T2DM was made according to the criteria of the World Health Organization (WHO).[10]

On admission (referred to as the baseline for the present investigation), participants underwent a routine medical history, physical examination and blood sampling. Information regarding smoking and family history of stroke was obtained using a standard questionnaire. Smoking habit was classified either as currently smoking or not currently smoking. Physical examination

Division of Nephrology and Hypertension, Department of Medicine, Diabetes Center, Tokyo Women's Medical University School of Medicine, Tokyo, Japan
Correspondence: Dr T Babazono, Division of Nephrology and Hypertension, Diabetes Center, Tokyo Women's Medical University School of Medicine, 8-1 Kawadacho, Shinjukuku, Tokyo 162-8666, Japan.
E-mail: babazono@dmc.twmu.ac.jp
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included blood pressure measurement and anthropometry, and laboratory examinations included hemoglobin A1C (A1C), serum lipids and creatinine levels using fasting blood samples, as well as urinary albumin excretion measured in the first morning urine specimen.

Clinical evidence of coronary artery disease (CAD) was defined as the presence of angina pectoris diagnosed by coronary angiography or myocardial scintigraphy, a history of myocardial infarction or previous coronary revascularization. A past history of peripheral artery disease (PAD) was defined as lower extremity PAD, according to the 2005 guidelines of the American College of Cardiology/American Heart Association.[11]

Measurements

Serum creatinine was initially measured in the hospital laboratory using Jaffé's method. In January 2003, Jaffé's method was replaced by an enzymatic method. Serum creatinine concentrations obtained after January 2003 were therefore adjusted using the following regression equation, which was obtained from a correlational analysis between both measurements of serum creatinine analysis in 10 132 samples from diabetic patients: serum creatinine (enzymatic method, mg per 100 ml)=0.972×serum creatinine (Jaffé's method, mg per 100 ml)−0.224 (r=0.9992, P<0.001). The GFR was estimated using the equation that originated from the Modification of diet in renal disease (MDRD) Study group equation,[12] refitted for Japanese individuals:[13] $GFR=175\times SCr^{-1.154}\times age^{-0.203}\times 0.742$ ([if female]×0.741), where SCr=serum creatinine measured by enzymatic methods in mg per 100 ml. Patients were divided into the following four categories by eGFR at baseline: ⩾90, 60–89, 30–59 and 15–29 ml min^{-1} per 1.73 m^2.

Classification of the degree of urinary albumin excretion was assessed according to the American Diabetes Association (ADA) criteria[14] on the basis of albumin-to-creatinine ratio (ACR) in the first morning urine specimen. Urinary ACR was calculated from urinary albumin, determined using the latex agglutination method and urinary creatinine concentration. Patients were classified into one of the following three categories: normoalbuminuria if the ACR was less than 30 mg per g Cr, microalbuminuria if the ACR was 30–299 mg per g Cr, or clinical albuminuria if the ACR was equal to or greater than 300 mg per g Cr.

A1C was determined by high-performance liquid chromatography (normal range: 4.3%–5.8%). Total cholesterol and high-density lipoprotein (HDL) cholesterol were determined enzymatically. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation when the plasma triglyceride level was less than 400 mg per 100 ml.

Outcomes

Patients were followed until September 2007. The primary end point was an incident first stroke event, the information regarding which was obtained by direct reference to medical records by a single investigator (R.B.). Stroke was defined as an acute focal neurological deficit lasting for longer than 24 h or resulting in death within 24 h of the onset of symptoms, which was diagnosed as attributable to a cerebral lesion of vascular origin [*International Classification of Diseases*, 9th Revision (ICD-9), codes of cerebrovascular diseases]. Most stroke cases were diagnosed by computed tomography, magnetic resonance imaging, including diffusion image and magnetic resonance angiography of the brain, and carotid duplex imaging.

Statistical analyses

Data were expressed as percentages, arithmetic means ± standard deviation (s.d.) or geometric means with a 95% confidence interval (CI) depending on the distribution of the data. Triglycerides, ACR and C-reactive protein (CRP) were logarithmically transformed because of skewed distributions. For statistical analyses, a one-way ANOVA was used for between-group comparisons for continuous variables and the Cochran-Armitage test was used for categorical variables. Kaplan-Meier curves were used to construct the cumulative incidence of stroke. The Cox proportional hazard model was used to estimate the HR and the 95% confidence interval (CI). All statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA), version 9.13. A P-value less than 0.05 was considered statistically significant.

RESULTS

A total of 1300 T2DM patients were enrolled between 2002 and 2003. The study included 546 women and 754 men, with a mean (± s.d.) age of 63 ± 13 years (range=21–92 years). At baseline, the mean eGFR was 70.7 ± 28.6 ml min^{-1} per 1.73 m^2 (range=15.4–155.5 ml min^{-1} per 1.73 m^2). A total of 290, 567, 335 and 108 diabetic patients were classified into the following eGFR-specific categories: ⩾90, 60–89, 30–59 and 15–29 ml min^{-1} per 1.73 m^2, respectively. Demographic and characteristic data regarding patients in the four eGFR categories are listed in Table 1. Compared with patients with an eGFR ⩾90, patients with an eGFR < 90 ml min^{-1} per 1.73 m^2 were likely to be older and to have a longer duration of diabetes and higher systolic but lower diastolic blood pressure, yielding higher pulse pressure. Furthermore, a decreased level of eGFR was significantly associated with a higher prevalence of diabetic retinopathy, CAD and PAD. Medications and laboratory data by eGFR categories are shown in Table 1 and Table 2, respectively.

During a mean follow-up period of 3.7 ± 1.4 years (median=4.0 years; maximum=5.5 years; overall=4784 patient-years), 91 first episodes of stroke were observed, yielding an incidence rate of 19.0 episodes per 1000 patient-years. We observed 77 stroke cases with cerebral infarction, 13 with cerebral bleeding and one with a subarachnoid hemorrhage.

As shown in Figure 1a, the 5-year cumulative incidence of stroke in patients with eGFRs ⩾90, 60–89, 30–59 and 15–29 ml min^{-1} per 1.73 m^2 was 7.0%, 7.9%, 14.7% and 15.5%, respectively (P=0.001 by log-rank test). Using a univariate Cox model (Figure 2a), the HRs of strokes for patients with eGFRs of 30–59 (2.35, 95% CI=1.28–4.30, P=0.006) and 15–29 ml min^{-1} per 1.73 m^2 (2.43, 95% CI=1.12–5.29, P=0.025) were significant compared with patients with an eGFR ⩾90 ml min^{-1} per 1.73 m^2.

In the multivariate Cox model analysis, the following variables were incorporated as candidates for explanatory variables: age, gender, body mass index, family history of stroke, smoking status, systolic and diastolic blood pressure, duration of diabetes, proliferative diabetic retinopathy, logarithmically transformed triglycerides, HDL/LDL cholesterol level, A1C, logarithmically transformed CRP, use of renin–angiotensin system blockers and antiplatelet medication, and logarithmically transformed urinary ACR. Stepwise selection was then applied to limit and indicate co-variables with a significant effect, selecting age (HR 1.06, 95% CI=1.03–1.08, P<0.001), current smoking status (HR 1.67, 95% CI=1.02–2.75, P=0.042), atrial fibrillation (HR 1.99, 95% CI=1.01–3.92, P=0.048) and LDL cholesterol (HR 1.01, 95% CI=1.00–1.01, P=0.022) as the final covariates. After adjusting for these significant variables, the significance of the HRs observed in the univariate analysis for patients with eGFRs of 30–59 and 15–29 ml min^{-1} per 1.73 m^2 disappeared (Figure 2b).

When participants were classified by urinary ACR at baseline (Figure 1b), the 5-year cumulative incidence of stroke in patients with normoalbuminuria, microalbuminuria and clinical albuminuria was 7.3%, 10.2% and 16.6%, respectively (P=0.0003 by log rank test). The HR of stroke events for patients with microalbuminuria (1.69, 95% CI=1.01–2.83, P=0.047, N=323) and clinical albuminuria (2.62, 95% CI=1.61–4.24, P<0.001, N=279) was significant in the univariate Cox model analysis when compared with patients with normoalbuminuria (N=698) (Figure 3a). In the multivariate Cox model that included the covariates listed above, but using eGFR instead of urinary ACR (Figure 3b), the HR for patients with clinical albuminuria remained significant (2.40, 95% CI 1.46–2.40, P<0.001). The significance found in patients with microalbuminuria, however, disappeared (1.41, 95% CI 0.83–2.37, P=0.202).

Table 1 Patients' characteristics and medications according to eGFR levels at baseline

eGFR (ml min^{-1} per 1.73 m^2)	*⩾ 90 (N=290)*	*60–89 (N=567)*	*30–59 (N=335)*	*15–29 (N=108)*	P-value[a]
Age (years)	56 ± 13	63 ± 12	68 ± 11	66 ± 13	<0.001
Gender (men, %)	56.2	57.1	59.1	63.9	0.167
Family history of stroke (%)	30.0	38.3	34.6	35.2	0.389
Current smoker (%)	27.9	22.4	19.4	25.9	0.089
Diabetes duration (years)	11 ± 8	14 ± 9	18 ± 10	18 ± 10	<0.001
Body mass index (kg m^{-2})	24.8 ± 4.8	24.3 ± 4.2	24.5 ± 3.8	24.3 ± 2.6	0.374
Systolic blood pressure (mm Hg)	129 ± 20	130 ± 19	134 ± 23	139 ± 26	<0.001
Diastolic blood pressure (mm Hg)	77 ± 11	75 ± 12	73 ± 13	76 ± 13	0.287
Pulse pressure (mmHg)	52 ± 17	55 ± 17	62 ± 19	63 ± 20	<0.001
Proliferative diabetic retinopathy (%)	24.1	29.8	40.6	58.3	0.001
Coronary artery disease (%)	5.9	12.2	23.0	21.3	<0.001
Atrial fibrillation (%)	4.1	4.1	8.4	2.8	0.209
Peripheral artery disease (%)	3.1	3.5	11.0	15.7	<0.001
Medication for diabetes (%)					
Oral	46.6	43.9	34.9	17.6	<0.001
Insulin	39.7	42.9	56.1	75.9	<0.001
Medication for hypertension (%)					
Ca channel blocker	21.7	30.5	42.1	72.2	<0.001
ACEI	16.2	16.9	27.2	26.9	<0.001
ARB	15.9	21.9	37.6	45.4	<0.001
β-Blocker	5.9	6.5	13.4	12.0	<0.001
α-Blocker	1.7	3.2	3.3	13.0	0.001
Antiplatelet drug (%)	16.9	29.8	45.7	44.4	<0.001
Statin (%)	23.1	23.8	38.2	21.3	0.010

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin type 1 receptor blocker; eGFR, estimated glomerular filtration rate.
Data are expressed as mean ± s.d. or percentage.
[a]ANOVA or Cochran–Armitage test.

Table 2 Laboratory data according to eGFR levels at baseline

eGFR (ml min^{-1} per 1.73 m^2)	*⩾ 90 (N=290)*	*60–89 (N=567)*	*30–59 (N=335)*	*15–29 (N=108)*	P-value[a]
A1C (%)	9.1 ± 1.9	8.6 ± 1.6	8.3 ± 1.8	7.5 ± 1.7	<0.001
Triglyceride (mg per 100 ml)[b]	117 (111–125)	120 (115–125)	139 (131–147)	135 (124–147)	0.075
HDL cholesterol (mg per 100 ml)	49 ± 15	48 ± 14	46 ± 15	41 ± 19	<0.001
LDL cholesterol (mg per 100 ml)	116 ± 35	115 ± 31	119 ± 38	115 ± 45	0.536
CRP (mg l^{-1})[b]	0.94 (0.83–1.07)	0.96 (0.88–1.04)	1.35 (1.21–1.52)	1.75 (1.41–2.18)	<0.001
Hemoglobin (g per 100 ml)	13.9 ± 1.6	13.7 ± 1.6	12.7 ± 1.6	10.8 ± 2.0	<0.001
ACR (mg per g Cr)[b]	22 (19–26)	25 (22–29)	93 (74–118)	842 (610–1163)	<0.001
<30 (%)	67.9	65.4	36.7	6.5	<0.001
30–299 (%)	26.9	24.2	28.1	13.0	0.128
⩾300 (%)	5.2	10.4	35.2	80.6	0.047
Serum creatinine (mg per 100 ml)	0.53 ± 0.10	0.72 ± 0.12	1.06 ± 0.22	2.33 ± 0.72	<0.001
eGFR (ml min^{-1} per 1.73 m^2)	108.7 ± 20.4	74.4 ± 8.5	47.8 ± 8.5	20.7 ± 5.5	<0.001

Abbreviations: ACR, albumin-to-creatinine ratio; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HDL/LDL, high/low-density lipoprotein.
Data are expressed as mean ± s.d., geometric mean (95% CI) or percentage.
[a]ANOVA or Cochran–Armitage test.
[b]Geometric mean.

Finally, HRs were calculated for six groups classified according to eGFRs ⩾60 ml min^{-1} per 1.73 m^2 or less and the degree of albuminuria to simultaneously determine the effects of these two renal manifestations on stroke events. The group with an eGFR⩾60 ml min^{-1} per 1.73 m^2 and normoalbuminuria was defined as the reference group. The impact of reduced eGFR on stroke was observed only in patients with clinical albuminuria, whereas a stepwise increase in HRs was identified as albuminuria increased, regardless of eGFR levels at baseline (Figure 4).

DISCUSSION

Although CKD has recently been identified as a serious risk factor for cardiovascular events, including stroke,[3,9,15] the independent effects of manifestations of CKD, albuminuria and reduced GFR have been largely unknown, especially in diabetic patients who carry a particularly high risk of developing stroke. To the best of our knowledge, this is the first study to determine the independent effect of eGFRs on the incidence of stroke events in T2DM patients, irrespective of the albuminuria levels. In this large hospital-based prospective cohort

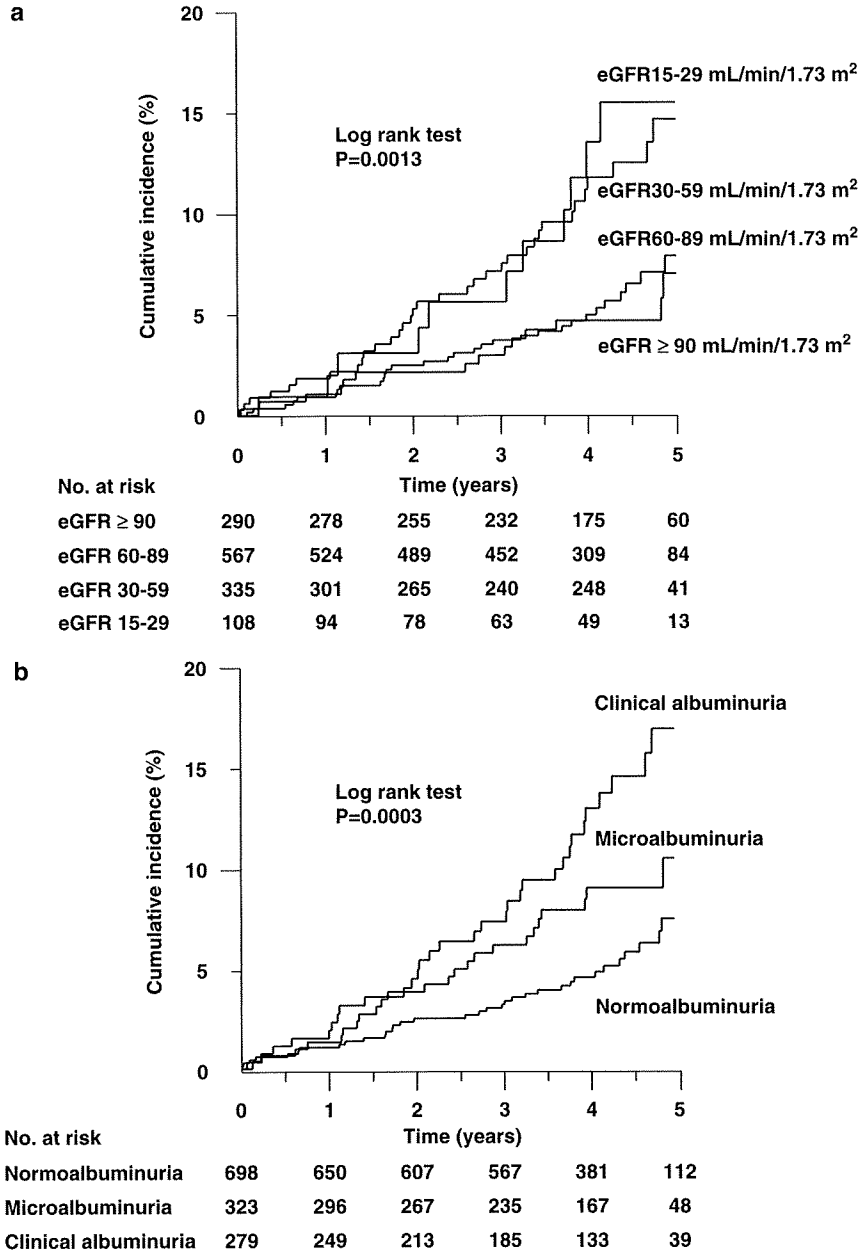

Figure 1 Kaplan–Meier curve of the cumulative incidence of first stroke events in type 2 diabetic patients stratified by estimated glomerular filtration rate (eGFR) (**a**) and urinary albumin (**b**). Normoalbuminuria, urinary albumin-to-creatinine ratio <30; microalbuminuria, 30–299; clinical albuminuria, ⩾300 mg per g Cr.

study of Japanese diabetic patients, we have shown that reduced eGFR is potentially associated with a higher risk of incident stroke in Japanese patients with T2DM; however, this association disappeared after adjusting for urinary ACR. In contrast, albuminuria, another renal manifestation of diabetic kidney disease, was a significant predictor of stroke events, even after adjusting for covariates such as eGFR.

In the recently published *post hoc* analyses from the Antihypertensive and Lipid-lowering Treatment to Prevent Heart Attack Trial (ALLHAT)[16] and the PROspective pioglitAzone Clinical Trial In macroVascular Events (PROactive),[17] more diabetic patients with CKD defined as having a reduced GFR, reached the primary composite cardiovascular end point than did patients without CKD. Unfortunately, the two studies lacked data regarding albuminuria/proteinuria, thereby limiting the understanding of the independent effect of reduced GFR. In addition, these clinical trials originally included both diabetic and non-diabetic individuals carrying a high cardiovascular risk. In contrast, our study included diabetic patients who had no history of stroke events, presumably yielding conflicting results in terms of the effect of GFR.

Post hoc analyses from the Irbesartan in Diabetic Nephropathy Trial (IDNT),[18] the Losartan Intervention For Endpoint reduction in

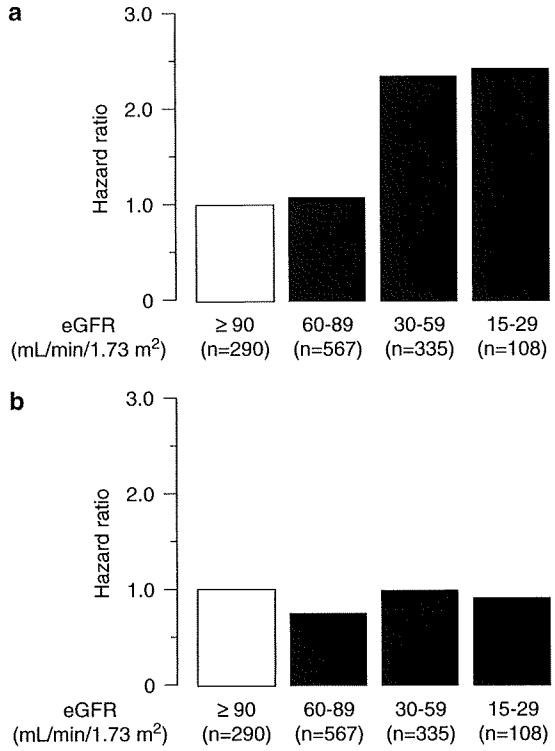

Figure 2 Hazard ratio of stroke in patients with type 2 diabetes in the univariate (**a**) and multivariate (**b**) Cox model stratified by eGFR levels (eGFR $\geqslant$90 versus eGFR 60–89, 30–59 and 15–29 ml min^{-1} per 1.73 m^2). The multivariate model included the following covariates: age, gender, body mass index, family history of stroke, smoking status, systolic blood pressure, diastolic blood pressure, duration of diabetes, proliferative retinopathy, hemoglobin A1C, logarithmically transformed triglyceride, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, logarithmically transformed C-reactive protein, use of renin–angiotensin system blockers, antiplatelet medication and a logarithmically transformed urinary albumin-to-creatinine ratio.

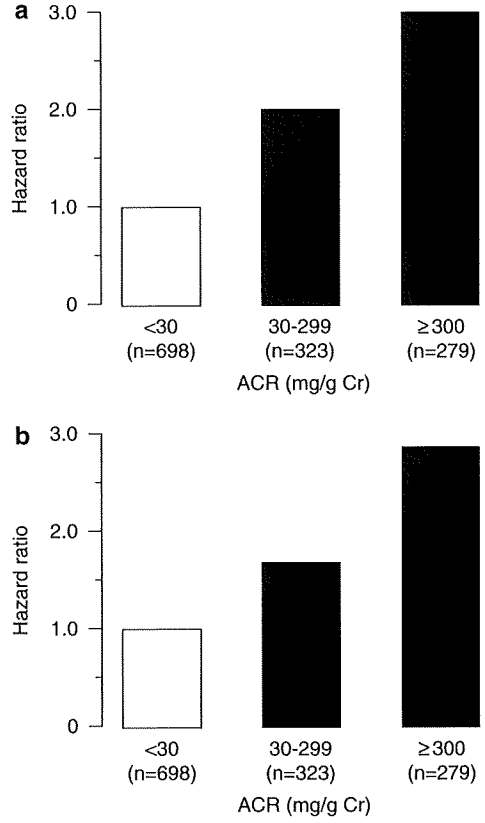

Figure 3 Hazard ratio of stroke in patients with type 2 diabetes in the univariate (**a**) and multivariate (**b**) Cox model stratified by urinary albumin-to-creatinine ratio. The multivariate model included the aforementioned covariates (see legend for Figure 2), but used eGFR instead of the urinary albumin-to-creatinine ratio.

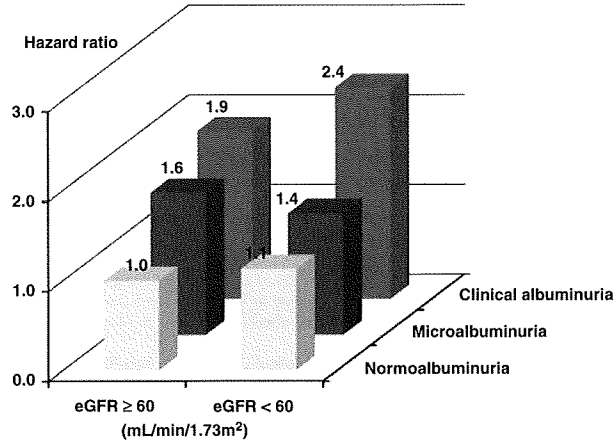

Figure 4 Hazard ratio of stroke for patients with type 2 diabetes in the multivariate model in six groups classified according to eGFR levels of $\geqslant$60 ml min^{-1} per 1.73 m^2 or less and the degree of albuminuria. The multivariate model included the aforementioned covariates (see legend for Figure 2), excluding the eGFR and the urinary albumin-to-creatinine ratio.

hypertension (LIFE) and the Reduction of Endpoints in NIDDM with the Angiotensin II Receptor Antagonist Losartan (RENAAL) studies,[19] as well as a population-based study from Hong Kong,[9] showed that both albuminuria and decreased values of GFR (or increased serum creatinine levels) were associated with a higher risk of composite cardiovascular end points in patients with T2DM. In these studies, however, the effects of renal parameters on the end point limited to stroke events were not determined.

The reason for the lack of association between reduced GFR and stroke is unclear. One possible explanation may be related to the heterogeneous pathogenesis of strokes. Most stroke cases are commonly classified as a brain hemorrhage, a subarachnoid hemorrhage or a brain infarction.[20] Brain infarctions are clinically subcategorized as atherothrombotic, cardioembolic and lacunar infarctions.[20] A recent study did not find a significant association between serum creatinine and lacunar infarction.[21] Almost half of the ischemic stroke cases were lacunar infarctions in our study, possibly attenuating the impact of eGFR on incident strokes. In addition, the smaller sample size in our study may limit the examination of the impact on each stroke subtype.

In contrast to reduced eGFR, clinical albuminuria was confirmed to be a strong and independent predictor of stroke events in our study cohort and of overall cardiovascular disease in earlier studies.[9,18,19,22–24] Albuminuria is a marker of renal and systemic vascular damage[25] that is associated with hypertension,[26] dyslipidemia[27] and obesity.[28] We

have shown earlier that albuminuria, but not a decreased eGFR, is independently associated with metabolic syndrome or abdominal obesity.[29] As the potency of albuminuria was maintained even after adjusting for these conventional risk factors in this study, non-traditional risk factors may serve as confounding factors. These may include inflammatory[30] and thrombogenic factors,[31] oxidative stress, homocysteine[32] and asymmetric dimethylarginine (ADMA),[33] all of which have been found to be associated with (micro)albuminuria in diabetic patients. A recent study has suggested that albuminuria, but not eGFR, was associated with arterial remodeling. This explains, at least in part, why albuminuria presents a greater risk of stroke.[34]

Our study has several limitations. First, this study was a hospital-based study, yielding a selection bias. Second, we studied only diabetic patients without earlier history of stroke to determine the effects of renal parameters in patients with T2DM on the first stroke event. Our results should therefore, be reviewed with caution when extrapolating the incidence of a secondary stroke event. Finally, albuminuria was determined from a single measurement of urinary ACR, possibly leading to improper categorization because of a marked day-to-day variability in albumin excretion. Although we did not obtain multiple measurements of urinary ACR, we restricted the timing of urine collection to the first morning to minimize exercise-induced and diurnal variation.[35]

In conclusion, our prospective hospital-based observational cohort study provides evidence that association between reduced eGFR and a higher risk of incident stroke observed in patients with T2DM may be due to underlying increases in albuminuria. The effects of albuminuria and eGFR on each stroke subtype and their ability to predict CAD, the other major cardiovascular event in patients with T2DM, remains to be elucidated.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cardiovascular, Pulmonary and Renal Pathology

Lowering Blood Pressure Blocks Mesangiolysis and Mesangial Nodules, but Not Tubulointerstitial Injury, in Diabetic eNOS Knockout Mice

Tomoki Kosugi,* Marcelo Heinig,* Takahiro Nakayama,* Thomas Connor,* Yukio Yuzawa,† Qiuhong Li,‡ William W. Hauswirth,‡ Maria B. Grant,§ Byron P. Croker,¶ Martha Campbell-Thompson,¶ Li Zhang,¶ Mark A. Atkinson,¶ Mark S. Segal,* and Takahiko Nakagawa*‖

From the Division of Nephrology, the Departments of Opthalmology,‡ and Pathology, Immunology and Laboratory Medicine,¶ and the Program in Stem Cell Biology,§ Department of Pharmacology and Therapeutics, University of Florida, Gainesville, Florida; the Department of Nephrology of Internal Medicine,† Nagoya University Graduate School of Medicine, Nagoya, Japan; and the Division of Renal Disease and Hypertension,‖ University of Colorado Denver, Aurora, Colorado*

Recently, we and others reported that diabetic endothelial nitric oxide synthase knockout (eNOSKO) mice develop advanced glomerular lesions that include mesangiolysis and nodular lesions. Interestingly, insulin treatment lowered blood pressure and prevented renal lesions, raising the question as to whether these beneficial effects of insulin were due to its ability to lower either high glucose levels or high blood pressure. We, therefore, examined the effect of lowering blood pressure using hydralazine in this diabetic eNOSKO mouse model. Hydralazine treatment significantly blocked the development of mesangiolysis and microaneurysms, whereas tubulointerstitial injury was not prevented in these mice. Additionally, hydralazine did not reduce expression levels of either tubulointerstitial thrombospondin-1 or transforming growth factor-β despite controlling blood pressure. On the other hand, the critical role of high glucose levels on the development of tubulointerstitial injury was suggested by the observation that serum glucose levels were correlated with tubulointerstitial injury, as well as with the expression levels of both transforming growth factor-β and thrombospondin-1. Importantly, controlling blood glucose with insulin completely blocked tubulointerstitial injury in diabetic eNOSKO mice. These data suggest that glomerular injury is dependent on systemic blood pressure, whereas hyperglycemia may have a more important role in tubulointerstitial injury, possibly due to the stimulation of the thrombospondin-1-transforming growth factor-β pathway in diabetic eNOSKO mice. This study could provide insights into the pathogenesis of advanced diabetic nephropathy in the presence of endothelial dysfunction. ***(Am J Pathol 2009, 174:1221–1229; DOI: 10.2353/ajpath.2009.080605)***

Diabetic nephropathy is pathologically characterized by glomerular hypertrophy, glomerular basement membrane thickening and mesangial expansion, and later by mesangiolysis and Kimmelstiel-Wilson nodules.[1,2] While numerous diabetic models have been able to reproduce the early mesangial changes, until recently, a model of advanced diabetic nephropathy has been lacking. Recently, we and others have reported that diabetic endothelial nitric oxide synthase knockout (eNOSKO) mice develop severe glomerular lesions, which resemble advanced lesions of human diabetic nephropathy.[1,2] Diabetic eNOSKO mice exhibit mesangiolysis, Kimmelstiel-Wilson-like nodules and glomerular capillary microaneurysms. Diabetic eNOSKO mice also develop worsening hypertension in association with renal injury.[1,2] Importantly, insulin treatment can control blood glucose, significantly reduce blood pressure, and prevent glomerular injury. This raises the question as to whether the beneficial effects of insulin on renal injury are due to controlling blood glucose and/or lowering blood pressure.

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Address reprint requests to Takahiko Nakagawa: Division of Renal disease and Hypertension, University of Colorado Denver, PO Box C281, Aurora, CO 80045. E-mail: takahiko.nakagawa@uchsc.edu.

Table 1. General Characteristics of Control and Diabetic Mice

	Non-DM		DM	
	Control	Hydralazine	Control	Hydralazine
Body weight (g)	27.9 ± 2.3	25.7 ± 4.7	21.5 ± 4.4*	24.1 ± 2.4
Blood glucose (mg/dl)	121 ± 7	111 ± 38	329 ± 63†	388 ± 56‡
K/B weight (10^{-3})	4.8 ± 0.6	5.3 ± 0.4	8.7 ± 1.4†	7.8 ± 2.0‡
BUN (mg/dl)	29 ± 3.0	32 ± 1.8	35 ± 5.6	32 ± 6.3
Ccr (ml/min)	0.13 ± 0.064	0.17 ± 0.082	0.048 ± 0.018*	0.089 ± 0.038§¶
Urine-albumin (μg/day)	73 ± 43	66 ± 18	273 ± 78‖	57 ± 31**
Urine-alb/Cre	0.16 ± 0.12	0.17 ± 0.07	13.2 ± 5.8‖	1.28 ± 1.18**

Non-DM, nondiabetic; K/B weight, kidney weight/body weight ratio; BUN, blood urea nitrogen; Ccr, creatinine clearance; Urine-alb/Cre, urine-albumin/creatinine.
*$P < 0.05$ vs Non-DM Control.
†$P < 0.01$ vs Non-DM Control.
‡$P < 0.01$ vs Non-DM +Hydralazine.
§$P < 0.05$ vs DM Control.
¶$P < 0.05$ vs Non-DM + Hydralazine.
‖$P < 0.005$ vs Non-DM Control.
**$P < 0.001$ vs DM Control.

Blood pressure control is considered a key recommendation for preventing the progression of diabetic renal disease.[3] However, the role of blood pressure control in the presence of endothelial dysfunction is not well understood. For example, Chen et al have examined the role of hypertension in apo E/eNOS double knockout mice and found that lowering blood pressure with hydralazine did not prevent the development of atherosclerosis and aneurysms.[4]

Given this finding, we examined if lowering in blood pressure through the use of hydralazine could block the development of advanced diabetic nephropathy, including glomerular and tubulointerstitial lesions, in the presence of endothelial dysfunction. In addition, we also evaluated the role of blood glucose on tubulointerstitial injury in this model.

Materials and Methods

Diabetes was induced in 8-week-old male C57BL/6J-Nos3tm1Unc (eNOSKO mice; Jackson Laboratory, Bar Harbor, ME) with intraperitoneal injections of streptozotocin (100 mg/dl/day for 2 consecutive days).[2] Blood glucose higher than 200 mg/dl was regarded as a diabetic state. A total of four groups with 12 mice per group were studied, including 1) non-diabetic diabetes mellitus (DM) eNOSKO, 2) non-DM eNOSKO with hydralazine, 3) DM-eNOSKO, and 4) DM-eNOSKO with hydralazine. Hydralazine was administered as 60 to 80 mg/kg body weight/day in the drinking water at 4 weeks. In addition, we reevaluated diabetic eNOSKO mice from our previous study to examine the effect of insulin on tubulointerstitial injury in this model (DM-eNOSKO with insulin treatment).[2] For blood sugar control, a single insulin pellet (Linshin Canada Inc, Ontario, Canada) was implanted subcutaneously for 5 months. Blood glucose was monitored every 2 weeks and if the fasting blood glucose was >200 mg/dl, an additional insulin pellet was inserted. Systolic blood pressure was assessed using a tail cuff sphygmomanometer (Visitech BP2000; Visitech Systems, Apex, NC). Blood urea nitrogen, urinary albumin excretion and urinary albumin/creatinine ratio were measured as described previously.[2] All animal experiments were performed in accordance with the Animal Care and Use Committee of the University of Florida.

Kidneys were fixed in Fekete's and embedded in paraffin for periodic acid-Schiff staining and immunohistochemistry. A polyclonal rabbit anti-human fibronectin antibody (1:200, Sigma-Aldrich, St. Louis, MO), polyclonal rabbit anti-mouse collagen IV antibody (Chemicon International, Temecula, CA), rabbit anti-human TGF- β polyclonal antibody (Santa Cruz biotechnology, Santa Cruz, CA), polyclonal goat anti-human collagen III antibody (Southern Biotechnology Associates, Birmingham, AL), polyclonal goat anti-rat vascular endothelial growth factor (VEGF) antibody (R&D Systems, Minneapolis, MN), mouse monoclonal thrombospondin-1 (TSP-1) antibody[5] and rat anti-mouse CD34 antibody[2] were used for immunohistochemistry. Negative controls were performed by the replacement of primary antibodies with species-matched antibodies.

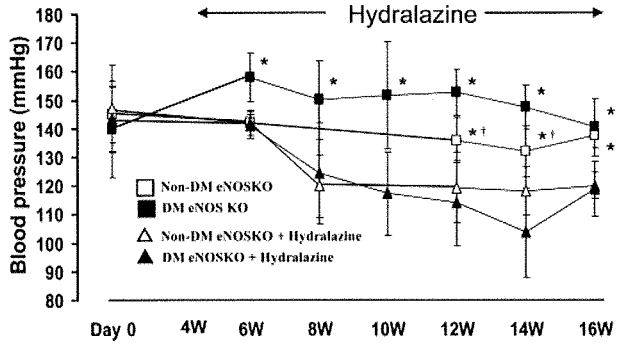

Figure 1. Time course of blood pressure in eNOS KO mice. Hydralazine treatment begins from 4 weeks. White square, non-diabetes; white triangle, non-diabetes with hydralazine treatment; black square, diabetes; black triangle, diabetes with hydralazine treatment. *$P < 0.01$ vs. diabetes with hydralazine treatment; †$P < 0.05$ vs. diabetes. n = 5/non-diabetes group. n = 10/diabetes group.

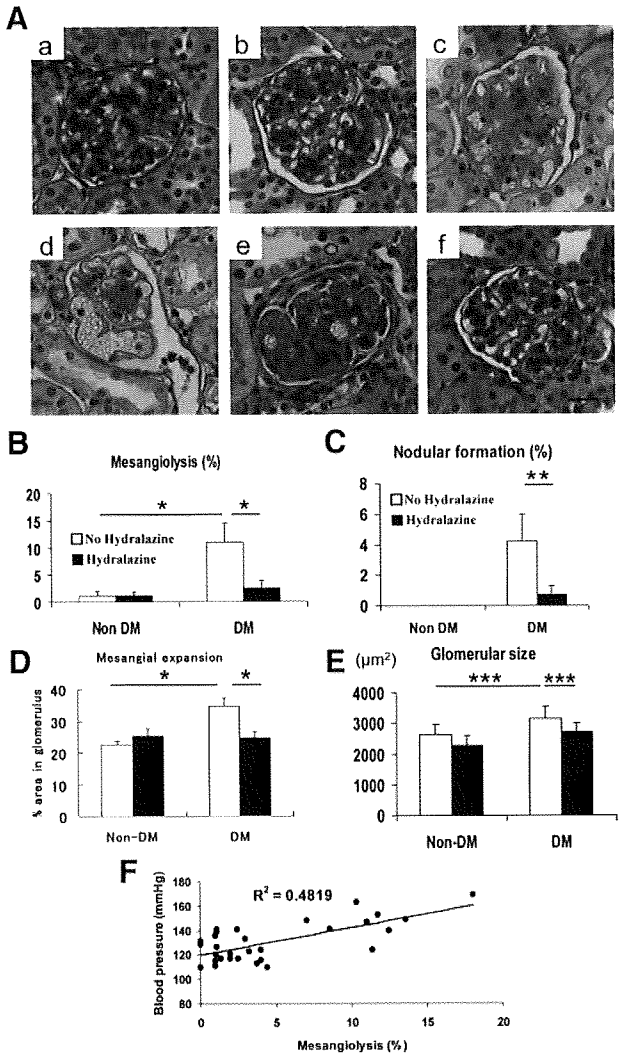

Figure 2. Glomerular lesions in diabetic eNOS KO mice. The representative glomerular injuries are shown (**A**). Compared with glomerulus in non-diabetes eNOSKO (**a**) and in non-diabetes with hydralazine (**b**), diabetes induces mesangial expansion (**c**), capillary microaneurysm (**d**), and nodular lesions (**e**). Hydralazine blocks the development of these lesions (**f**). Scale bar = 10 μm. Diabetic condition induces mesangiolysis (**B**), nodular lesions (**C**), mesangial expansion (**D**), and glomerular hypertrophy (**E**), all of which are blocked by hydralazine treatment. The development of mesangiolysis is correlated with blood pressure (**F**). Data are shown as means and SD. $^{*}P < 0.001$; $^{**}P < 0.01$; $^{***}P < 0.05$. n = 5/non-diabetes group. n = 10/diabetes group.

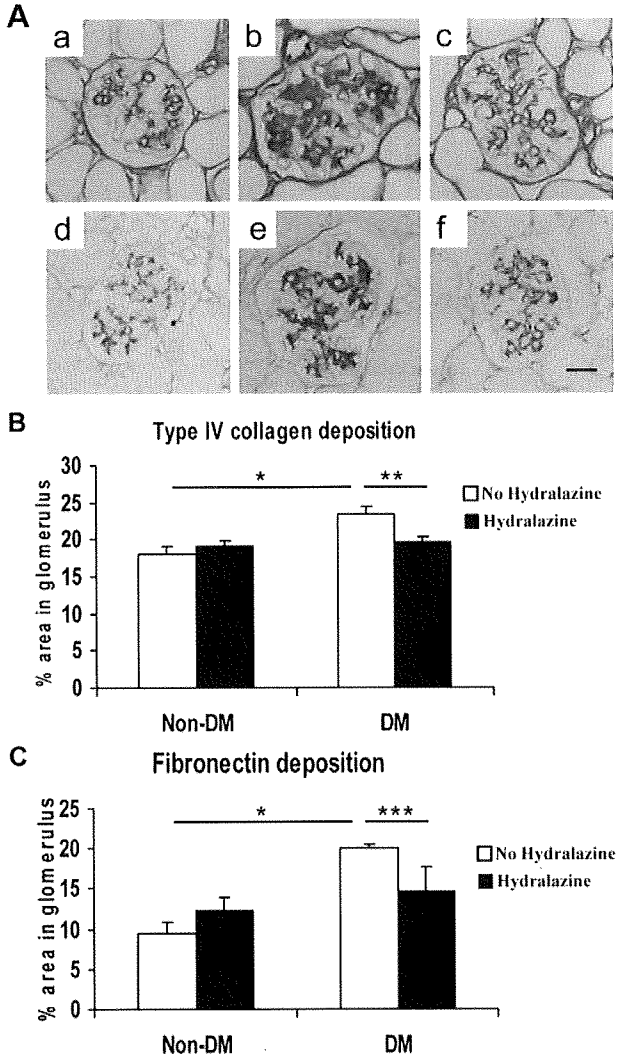

Figure 3. The depositions of extracellular matrix in glomeruli. The representative features of extracellular matrix deposition in glomerulus are shown (**A**). Immunohistochemical staining shows the pattern of collagen IV (**a, b, c**) and fibronectin (**d, e, f**) depositions. Compared with the non-diabetic condition (**a, d**), diabetes increases the collagen IV (**d**) and fibronectin (**e**) deposition, both of which are blocked by hydralazine treatment (**c, f**). Bar: 10 μm. Quantitative analysis is shown for collagen IV deposition (**B**) and fibronectin (**C**). Data are shown as means and SD. $^{*}P < 0.001$; $^{**}P < 0.005$; $^{***}P < 0.05$. n = 5/non-diabetes group. n = 10/diabetes group.

The mesangial area was determined by assessing the periodic acid-Schiff-positive and nuclei-free area in the mesangium. The glomerular area was also treated along the outline of capillary loop. These areas were measured using the AxioVision image analysis computer program (Carl Zeiss, Thrornwood, NY). Semiquantitative analysis for glomerular mesangiolysis was performed with 100 glomeruli in randomly selected fields for each subject. Mesangiolysis and nodular formation was assessed by scoring 100 glomeruli/mouse. The percentage of atrophic tubules (ie, tubular dilation, detachment of tubular epithelial cells, and condensation of tubular nuclei) was assessed by scoring 400 renal cortical tubules in randomly selected fields for each subject.[6] All quantifications were performed in a blinded manner by two independent investigators.

Real-Time PCR

The mRNA extraction and cDNA synthesis were performed by using an RNeasy Mini kit and QuantiTect Reverse Transcription Kit (Qiagen Science, Valencia, CA) according to the manufacturer's instructions. Real time PCR was performed for VEGF mRNA expression with whole kidneys as described previously.[2]

Western Blot Analysis

Whole mouse kidneys were snap-frozen in liquid nitrogen for protein isolation. Western blot analysis was performed

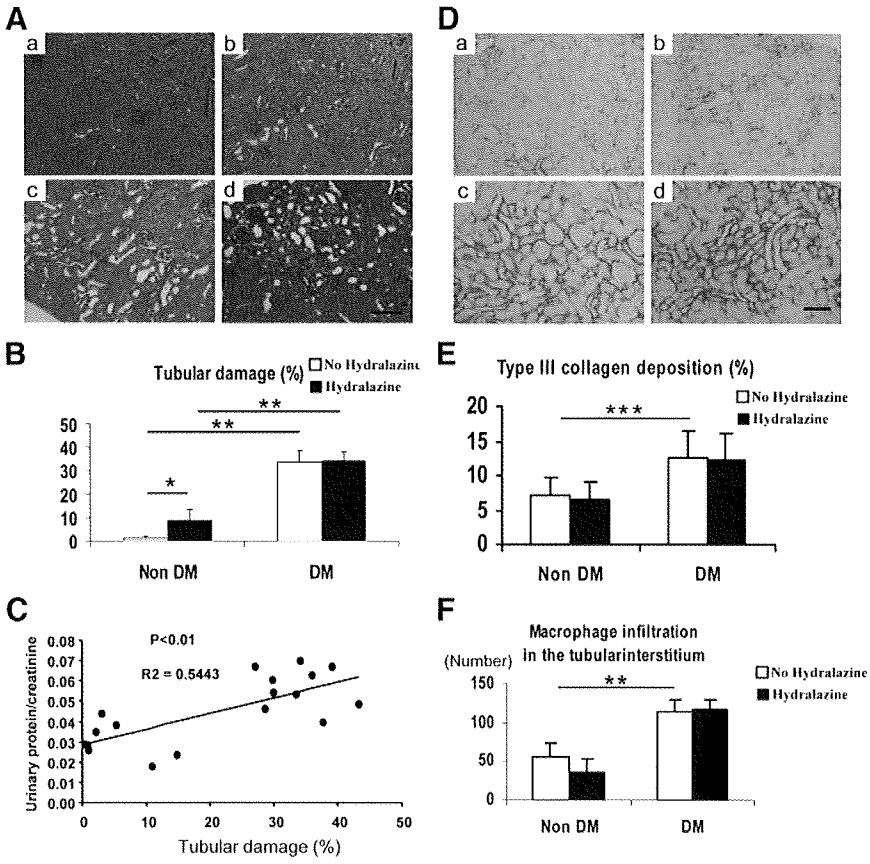

Figure 4. Tubulointerstitial injury in diabetic eNOS KO mice. In periodic acid-Schiff staining, the representative features of tubulointerstitial injury in non-diabetes (**a**), non-diabetes with hydralazine (**b**), diabetes (**c**) and diabetes with hydralazine (**d**) are shown (**A**). Scale bar = 50 μm. The quantitative analysis for tubulointerstitial injury with periodic acid-Schiff staining shows that diabetes induces tubular damage, which is not prevented by hydralazine treatment (**B**). Tubular injury is positively correlated with urinary protein excretion (urinary protein/urinary creatinine) (**C**). Type III collagen deposition in non-diabetes (**a**), non-diabetes with hydralazine (**b**), diabetes (**c**) and diabetes with hydralazine (**d**) are shown (**D**). Scale bar = 50 μm. It is quantitatively demonstrated that type III collagen deposition (**E**) and F4/80-positive macrophage infiltration (**F**) are prominent in diabetes while hydralazine has no effect. Data are shown as means and SD. $^{*}P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.05$. $n = 10$/group.

as described previously.[7] The blots were subsequently incubated with a polyclonal goat anti-rat VEGF antibody, mouse monoclonal TSP-1 antibody, or a monoclonal anti-mouse β-actin antibody (Sigma-Aldrich, St. Louis, MO), followed by incubation with peroxidase-conjugated rabbit IgG, goat IgG, or mouse IgG (DakoCytomation, Carpinteria, CA). Proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia, Piscataway NJ). The density of each band was measured using the public domain NIH Image program.

Statistical Analysis

All values are expressed as mean ± SD. Statistical analysis was performed with unpaired, two-tailed Student's *t*-test for single comparisons or analysis of variance with posthoc test using Tukey's method for multiple comparisons. A *P* value of <0.05 was considered significant.

Results

General Characteristics

As shown in Table 1, streptozotocin induced hyperglycemia in eNOSKO mice. Hydralazine did not alter the level of blood glucose in these mice. The diabetic state resulted in body weight loss, but interestingly, weight loss was noted to occur to a lesser degree in the hydralazine-treated group, despite the high levels of blood glucose. Diabetes induced renal hypertrophy (as evidenced by kidney/body weight ratio) was not completely blocked by hydralazine. In terms of renal function, diabetes significantly reduced creatinine clearance (Ccr) while Blood urea nitrogen level tended to be increased. However, Ccr was partially, but significantly improved by hydralazine treatment. Similarly, the high level of urinary albumin excretion observed in DM-eNOSKO mice was significantly blocked by hydralazine treatment.

Blood Pressure

Blood pressure was elevated in diabetic eNOSKO mice as early as 6 weeks and lasted for 10 weeks (Figure 1). Hydralazine markedly reduced blood pressure to levels as low as 100mmHg, which was lower than that observed in the non-diabetic eNOSKO. This blood pressure level was equivalent to that present in non-diabetic wild-type mice (data not shown).

Glomerular Histology

Compatible with previous reports,[1,2] diabetic eNOSKO mice developed early lesions, including mesangial expansion and collagen deposition, as well as advanced lesions, such as mesangiolysis, glomerular microaneurysms and Kimmelstiel-Wilson-like nodular lesions (Figure 2A). Interestingly, hydralazine markedly prevented

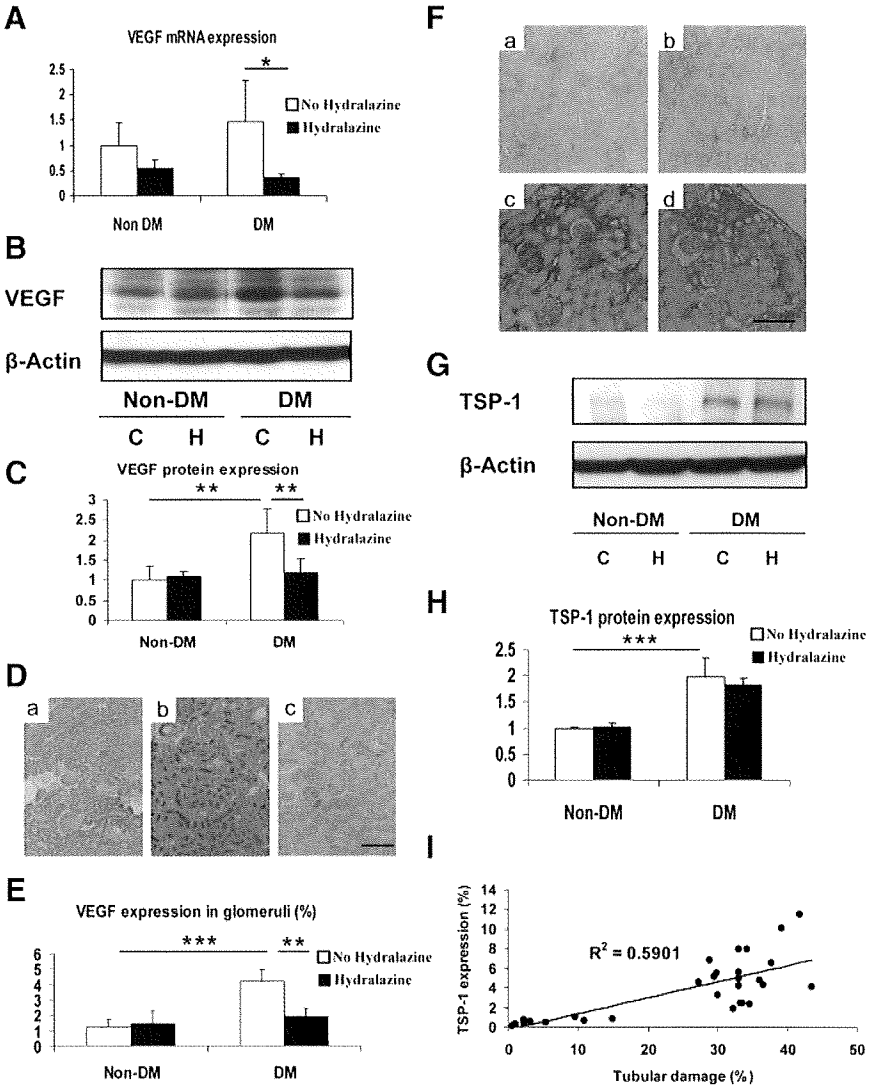

Figure 5. VEGF and TSP-1 expressions in diabetic eNOS KO mice. Real-time PCR for VEGF mRNA (**A**) and Western blotting for VEGF protein (**B, C**) in whole kidney demonstrated that VEGF expression is increased in diabetic eNOSKO mice. However, an increase in VEGF expression in diabetic eNOSKO mice is prevented by hydralazine treatment. In **C**, the quantitative result is expressed as the relative ratio of VEGF to β-actin. In **D** (scale bar = 50 μm), VEGF protein expression in immunohistochemistry demonstrated that compared with non-diabetic animal (**a**), diabetes exhibits intensive signal for VEGF in apical membrane of tubular cells (**b**). This intensive signal cannot be observed in diabetic eNOSKO mice with hydralazine treatment (**c**). Quantitative analysis shows that VEGF expression in glomeruli is high in diabetes, but it is significantly blocked by hydralazine treatment (**E**). **F:** TSP-1 protein expression is examined using immunohistochemistry (scale bar = 20 μm). TSP-1 protein expression is examined using immunohistochemistry (scale bar = 20 μm) (**F**). Compared with non-diabetic eNOSKO mice (**a**) or non-diabetic eNOSKO mice with hydrazaline treatment (**b**), diabetic eNOSKO mice exhibit intensive signal for TSP-1 in tubulointerstitium (**c**). This intensive signal does not demonstrate any difference in diabetic eNOSKO mice with hydrazaline treatment (**d**) compared with diabetic eNOSKO mice (**c**). Western blotting demonstrates the upregulation of TSP-1 in whole kidney in diabetic eNOSKO mice, but hydralazine has no effect on TSP-1 expression (**G**). TSP-1 protein expression is quantified with Western blotting (**H**). In **G**, the quantitative result is expressed as the relative ratio of TSP-1 to β-actin. Tubulointerstitial injury is positively correlated with TSP-1 expression in all groups (**I**). Data are shown as means and SD $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. n = 5/non-diabetes group. n = 10/diabetes group.

the development of these lesions, despite the presence of high glucose (Figure 2, B and C). The finding that mesangiolysis positively correlated with blood pressure suggests that the development of mesangiolysis was dependent on blood pressure in this model (Figure 2F). In addition, the early lesions, including glomerular hypertrophy, mesangial expansion, and the deposition of extracellular matrix, were also prevented by hydralazine (Figure 2, D and E). On the contrary, hydralazine did not alter glomerular morphology in non-diabetic mice (Figure 2A).

The Deposition of Extracellular Matrix in Glomerulus

Diabetes resulted in increased collagen IV and fibronectin deposition in the mesangium of eNOSKO mice. These changes were significantly prevented by hydralazine treatment (Figure 3, A–C).

Tubulointerstitial Histology

Although lowering blood pressure prevented the development of glomerular injury, renal function was not completely improved. Therefore, we examined the tubulointerstitial injury that occurs in this model. As shown in Figure 4A, diabetic eNOSKO mice developed severe tubular damages with tubular dilation and detachment of tubular epithelial cells, suggesting that tubulointerstitial injury was not dependent on blood pressure (Figure 4, A and B). In fact, no correlation was observed between blood pressure and tubular damage (data not shown) while tubular injury correlated with urinary protein excretion (Figure 4C).

To confirm tubulointerstitial injury, we examined type III collagen deposition and macrophage infiltration. Diabetic eNOSKO mice showed increased tubulointerstitial collagen III deposition, which was not improved by hydralazine (Figure 4, D and E). Similarly, while F4/80 positive macrophage infiltration in the tubuloint-

erstitium was prominent in the diabetic condition, hydralazine had no effect (Figure 4F). These data also suggest that the tubulointerstitial damage was not dependent on blood pressure in this model. Importantly, the tubulointerstitial injury could account for the impaired renal function despite controlled blood pressure.

Role of VEGF, TSP-1, and TGF-β in Tubulointerstitial Injury

We next attempted to seek the potential mechanisms for the tubulointerstitial injury. First, we investigated VEGF and TSP-1 expression, both of which are important pathogenic mediators of diabetic nephropathy. As shown in Figure 5, A–C, VEGF mRNA and protein expression were increased in the whole kidney of diabetic eNOSKO mice. VEGF was predominantly located in apical membrane in tubular epithelial cells (Figure 5D) as well as glomerular podocyte (data not shown). Interestingly, hydralazine treatment significantly reduced VEGF expression in the tubules and glomeruli (Figure 5E), suggesting that VEGF could be a mediator for glomerular injury, but not tubulointerstitial injury in this model.

TSP-1 expression was also induced in the diabetic eNOSKO mice as evidenced by immunohistochemistry (Figure 5F) and Western blotting (Figure 5, G and H). However, TSP-1 expression, in contrast to VEGF, was not prevented by hydralazine treatment. Furthermore, TSP-1 expression correlated with tubulointerstitial injury (Figure 5I), suggesting that TSP-1 could have a role in the tubulointerstitial injury independently of blood pressure in this model.

Since TSP-1 is known to activate TGF-β, which is one of the most important mediators in diabetic nephropathy, we next examined the role of TGF-β in this model. As shown in Figure 6, A–B, TGF-β expression was up-regulated in damaged tubules in diabetic mice. Hydralazine did not prevent TGF-β induction despite controlling blood pressure. Finally, TGF-β expression correlated with tubulointerstitial injury (Figure 6C), and with TSP-1 expression (Figure 6D). These data suggest that tubulointerstitial injury may be mediated by TGF-β, which could be activated by TSP-1.

Role of Glucose in Tubulointerstitial Injury

The evidence that hydralazine did not improve tubulointerstitial injury indicates that this form of injury might occur secondary to hyperglycemia in this model. Compatible with our assumption, we found that the tubulointerstitial injury in this model was positively correlated with blood glucose level (Figure 7A). Interestingly, TGF-β as well as TSP-1 expression correlated with blood glucose (Figure 7, B and C), suggesting glucose may be responsible for the tubulointerstitial injury via TSP-1-TGF-β pathway. To confirm this finding, we revisited our previous study in which diabetic eNOSKO mice were treated by insulin to maintain normal glucose level (Table 2). Histological analysis demonstrated that insulin treatment significantly prevented the development of tubulointerstitial injury along with a reduction in macrophage infiltration (Figure 7, D and E). These data suggest that tubulointerstitial injury in this model is likely mediated by blood glucose, but not blood pressure.

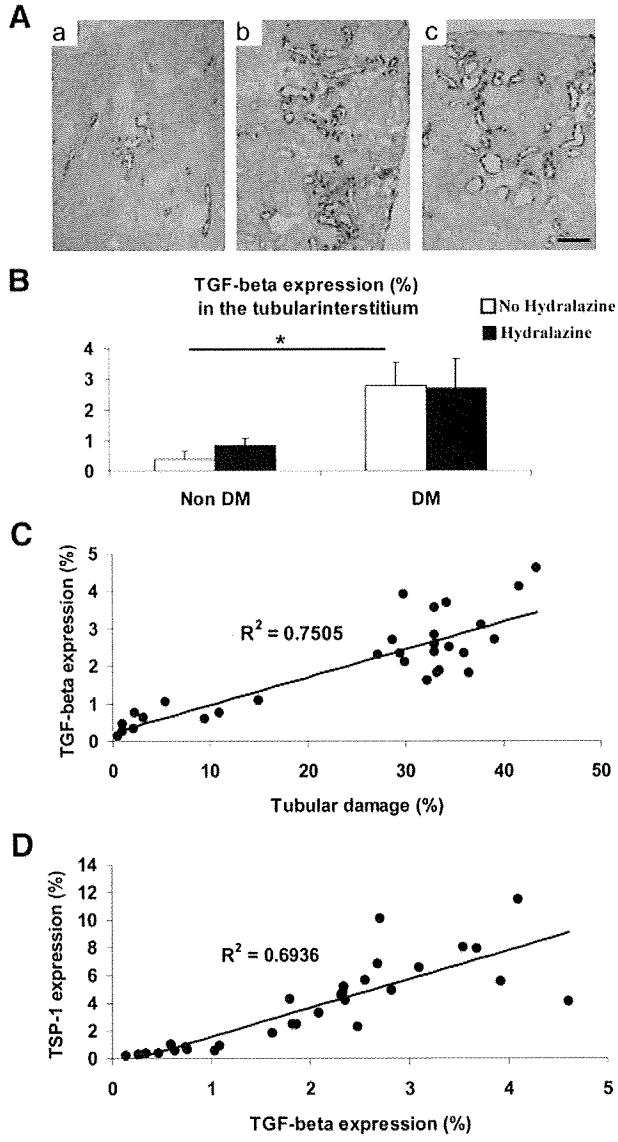

Figure 6. A: Tubular TGF-β expression in diabetic eNOS KO mice. Tubular TGF-β protein expression is examined using immunohistochemistry (scale bar = 20 μm). Compared with non-diabetic animals (**a**), diabetic mice exhibit intensive signal for TGF-β in damaged tubular cells (**b**). **B:** This intensive signal does not show any difference between diabetic eNOSKO mice with hydralazine treatment and diabetic eNOSKO mice (c). TGF-β protein expression is significantly increased in diabetic eNOSKO mice with/without hydralazine treatment in immunohistochemistry. TGF-β positively correlates with tubulointerstitial injury (**C**) and with TSP-1 expression (**D**) in all groups. Data are shown as means and SD. $^*P < 0.01$. $n = 5$ for non-DM group. $n = 10$ for DM group.

Discussion

The major finding in this study is the histological evidence that lowering blood pressure blocked the development of advanced diabetic glomerular injury in the presence of

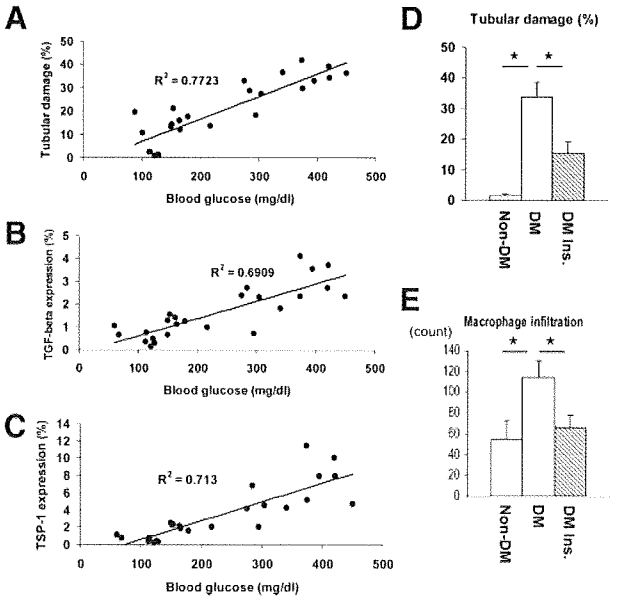

Figure 7. The effect of high glucose on tubulointerstitial injury in diabetic eNOS KO mice. Blood glucose positively correlates with tubulointerstitial injury (**A**), TGF-β expression (**B**), and TSP-1 expression (**C**) in non-DM (n = 5), DM (n = 10), and DM-Ins (Insulin treatment) (n = 10) groups. Insulin treatment significantly blocks the development of tubulointerstitial injury (**D**) as well as macrophage infiltration in tubulointerstitium (**E**). Data are shown as means and SD, $^*P < 0.05$.

eNOS deficiency. On the other hand, the tubulointerstitial injury was not dependent on blood pressure, and appeared to be due to the effects of hyperglycemia to stimulate the TSP-1-TGF-β pathway. A marked reduction of Ccr in diabetic eNOSKO was also observed. While it is possible that the reduction in Ccr could be secondary to dehydration (from the glycosuria), the fact that hydralazine treatment improved the renal function suggests that the reduced GFR was likely due to the renal injury.

Our study confirms a key role for blood pressure control to protect the glomerulus from diabetic injury in the eNOSKO mice. This protection could be potentially due to an improvement in glomerular hemodymanics. While controlling blood pressure was protective, it is also known that most hypertensive animal models do not produce mesangiolysis and Kimmelstiel-Wilson-like nodular lesions.[8,9] Previous studies have suggested that endothelial dysfunction may alter autoregulation, and consequently predispose the animal to glomerular hypertension.[10,11] In this process, endothelial NO is an indispensable factor to regulate the intraglomerular pressure by modulating afferent and efferent arterioles.[12,13] This could also explain why only a mild elevation of blood pressure (15–20 mmHg) observed in this model could have such a strong impact on the glomerulus to cause advanced lesions. This is also indicating that lowering blood pressure may help prevent glomerular injury, and indeed targeting lower blood pressures may be required in this setting. In addition, endothelial dysfunction was shown to be associated with glomerular injury, including glomerular microangiopathy and mesangiolysis, in other animal models.[14,15] These studies implicate the importance of endothelial dysfunction as an accelerating factor for renal injury.

Despite the importance of blood pressure on the glomerular injury in this model, we found that blood pressure control did not improve the tubulointerstitial damage even though albuminuria was reduced. In contrast, tubulointerstitial injury correlated with blood glucose levels. Glucose is known to enter tubular cells via Glut-1, an insulin-independent glucose transporter.[16] Therefore, a high level of glucose could theoretically stimulate tubular cells in the absence of insulin, causing tubulointerstitial injury independently of blood pressure. Consequently, high glucose can stimulate the expressions of TSP-1 and TGF-β in tubulointerstitium.[17–19] In addition, Wang et al demonstrated that glucose-induced TGF-β/TSP-1 expression was negatively regulated by nitric oxide in mesangial cell.[20] Given that, NO deficiency might contribute to tubular TGF-β and TSP-1 expression in this model.

An alternative possibility is that the tubulointerstitial injury occurred secondary to the streptozotocin, as streptozotocin is known to be nephrotoxic. However, the observation that the tubulointerstitial injury correlated with blood glucose levels and that insulin treatment improved the glucose levels in concert with reducing the renal injury suggests that the renal injury was secondary to the diabetic state. Alternatively, it is possible that insulin could be working via actions independent of glucose. For example, insulin can stimulate nitric oxide release from endothelial cells. Tubular cells also possess the insulin receptor and insulin can exert a variety of actions in tubular cells, including an inhibition of gluconeogenesis and modulation of sodium and phosphate transport.[21] Recently a protective role of insulin in tubular cells has been shown, as insulin stimulates PI3-Kinase/AKT pathway and inhibits apoptosis.[22]

TGF-β is one of the most important mediators in diabetic nephropathy.[23,24] However, TGF-β, which is secreted as an inactive form, can be converted to its active form by TSP-1. By binding to both the latency-associated protein and the mature TGF-β, TSP-1 induces the conformational changes of TGF-β, which allows it to bind its receptor.[25,26] Recently, Daniel et al examined the role of TSP-1 in diabetic TSP-1 knockout mice.[27] They found that the development of diabetic nephropathy was significantly attenuated in these mice. Interestingly, although TGF-β expression was increased, its activation was blocked in these mice, confirming that the TGF-β activation is mediated by TSP-1. On the other hand, we have previously demonstrated that TGF-β, in turn, stimulates TSP-1 expression via Smad2 activation in proximal tubular cells.[5] Thus, once tubular cells are stimulated by

Table 2. General Characteristics of Diabetic eNOS KO Mice with Insulin at 3 Months

	Body weight	Blood glucose	Systemic BP	K/B weight (10^{-3})	BUN
DM with insulin	26.2 ± 2.2	90 ± 40	138 ± 14	4.8 ± 0.7	17.1 ± 3.0

high glucose, both TGF-β and TSP-1 are induced and likely create a vicious cycle to induce tubulointerstitial injury *in vivo*.

In this study, we found that tubular VEGF expression was enhanced in diabetic conditions, but the behavior of VEGF was different from that observed with TSP-1/TGF-β. In fact, lowering blood pressure reduced VEGF expression. These data suggests that tubular VEGF expression is regulated by blood pressure, but not glucose. The association of VEGF expression with blood pressure has been shown in the DOCA salt hypertensive rats in which tubular VEGF expression was also reduced by lowering blood pressure with spironolactone.[28] Recently Advani et al demonstrated that renal VEGF expression was upregulated in spontaneously hypertensive rat and transgenic (mRen-2)27 rats.[29] Given the fact that VEGF121 administration lowered blood pressure in the rat,[30] the induction of VEGF could be a compensatory mechanism in response to hypertension in these animal models.

While VEGF may not mediate tubulointerstitial injury in this model, it is known that VEGF mediates early diabetic nephropathy in several diabetic animal models.[31,32] The association of renal VEGF with human diabetic nephropathy is also documented.[33,34] Recent evidence has demonstrated that the balance between VEGF and TSP-1 could be important to determine the fate of renal injury.[35,36] Compatibly, we found that hydralazine reduced tubular VEGF expression while TSP-1 expression in tubulointerstitial lesion was still sustained. Therefore, the imbalance of VEGF with TSP-1 may account for the tubulointerstitial injury in this model.

In conclusion, our study demonstrates that in the presence of endothelial dysfunction, blood pressure is critical for the development of glomerular lesions while blood glucose may be more critical as a mediator of tubulointerstitial injury. Nevertheless, insulin treatment was able to prevent both lesions. These results may provide insights into the effects of these key treatments on diabetic nephropathy in humans.

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The growth factor midkine regulates the renin-angiotensin system in mice

Akinori Hobo,[1,2] Yukio Yuzawa,[2] Tomoki Kosugi,[2] Noritoshi Kato,[1,2] Naoto Asai,[2] Waichi Sato,[2] Shoichi Maruyama,[2] Yasuhiko Ito,[2] Hiroyuki Kobori,[3] Shinya Ikematsu,[4] Akira Nishiyama,[5] Seiichi Matsuo,[2] and Kenji Kadomatsu[1]

[1]Department of Biochemistry and [2]Department of Nephrology, Nagoya University Graduate School of Medicine, Nagoya, Japan.
[3]Department of Physiology and Hypertension & Renal Center of Excellence, Tulane University Health Sciences Center, New Orleans, Louisiana, USA.
[4]Department of Bioresources Engineering, Okinawa National College of Technology, Okinawa, Japan.
[5]Department of Pharmacology and Hypertension & Kidney Disease Research Center, Kagawa University Medical School, Kagawa, Japan.

The renin-angiotensin system plays a pivotal role in regulating blood pressure and is involved in the pathogenesis of kidney disorders and other diseases. Here, we report that the growth factor midkine is what we believe to be a novel regulator of the renin-angiotensin system. The hypertension induced in mice by 5/6 nephrectomy was accompanied by renal damage and elevated plasma angiotensin II levels and was ameliorated by an angiotensin-converting enzyme (ACE) inhibitor and an angiotensin receptor blocker. Notably, ACE activity in the lung, midkine expression in the lung, and midkine levels in the plasma were all increased after 5/6 nephrectomy. Exposure to midkine protein enhanced ACE expression in primary cultured human lung microvascular endothelial cells. Furthermore, hypertension was not induced and renal damage was less severe in midkine-deficient mice. Supplemental administration of midkine protein to midkine-deficient mice restored ACE expression in the lung and hypertension after 5/6 nephrectomy. Oxidative stress might be involved in midkine expression, since expression of NADH/NADPH oxidase–1, –2, and –4 was induced in the lung after 5/6 nephrectomy. Indeed, the antioxidative reagent tempol reduced midkine expression and plasma angiotensin II levels and consequently ameliorated hypertension. These results suggest that midkine regulates the renin-angiotensin system and mediates the kidney-lung interaction after 5/6 nephrectomy.

Introduction

The renin-angiotensin system (RAS) is a hormonal cascade that functions in the homeostatic control of arterial pressure, tissue perfusion, and extracellular volume. Dysregulation of the RAS results in the pathogenesis of many diseases, including cardiovascular and renal disorders (1–3). The RAS is initiated by the regulated secretion of renin, which catalyzes the hydrolysis of Ang I from the N terminus of angiotensinogen. Ang I is in turn hydrolyzed by angiotensin-converting enzyme (ACE) to form Ang II, the primary active product of the RAS (4, 5). ACE is a zinc metallopeptidase widely distributed on the cell membrane of endothelial and epithelial cells (6). Ang II induces vasoconstriction and aldosterone release, leading to upregulation of blood pressure. It also exerts its vasoconstrictor effect on both the afferent and efferent arterioles, which may contribute to the onset and progression of chronic renal damage. Ang II may also directly contribute to the acceleration of renal damage by sustaining cell growth, inflammation, and fibrosis (7).

The growth factor midkine (MK; gene symbol, *MDK*) is implicated in cancer progression, neuronal survival and differentiation, and inflammation (8). MK is involved in the pathogeneses of tubulointerstitial damage induced by renal reperfusion and glomerular sclerosis associated with diabetes mellitus (9, 10). The finding of a recent report that angiotensinogen and renin expression was significantly elevated in the aorta of $Mdk^{-/-}$ mice while ACE expression was significantly suppressed is of particular interest (11). However, $Mdk^{-/-}$ mice develop normally (8), and there has been no report of systemic disturbance or organ disorders of $Mdk^{-/-}$ mice. Therefore, the biological meaning of changes in the RAS molecules in the aorta of $Mdk^{-/-}$ mice has remained obscure.

It is widely accepted that the RAS is involved in the pathogenesis of chronic kidney disease (CKD), and inhibitors of the RAS are the first choice of therapy for CKD (12–14). To investigate the molecular mechanisms regulating the RAS in CKD, we employed 5/6 nephrectomy in this study. 5/6 nephrectomized mice are a popular and useful model of CKD, since the remnant kidney model of progressive renal injury is characterized by systemic hypertension and glomerular hyperfiltration, the latter eventually causing glomerular sclerosis (15, 16). CKD accompanies multiple organ failure, the pathogenesis of which involves inter-organ cross-talk (17, 18). In this context, it is noteworthy that MK expression was induced in the lung by 5/6 nephrectomy, leading to elevation of ACE activity and plasma Ang II levels and subsequent hypertension in the present study. Our data therefore suggest that MK is a candidate mediator of inter-organ cross-talk in CKD.

Results

MK is involved in RAS activation induced by 5/6 nephrectomy. Systolic and mean blood pressure were comparable in untreated $Mdk^{+/+}$ and $Mdk^{-/-}$ mice (Figure 1, A and B). However, we found that 5/6 nephrectomy strikingly increased blood pressure in $Mdk^{+/+}$ mice but not in $Mdk^{-/-}$ mice (Figure 1, A and B). The systolic and mean blood pressure of $Mdk^{+/+}$ mice strikingly increased after 2 weeks, but $Mdk^{-/-}$ mice showed almost normal blood pressure, i.e., no

Conflict of interest: The authors have declared that no conflict interest exists.

Nonstandard abbreviations used: ACE, angiotensin-converting enzyme; BIS, bisindolylmaleimide I; CKD, chronic kidney disease; HMVEC-L, human lung microvascular endothelial cell(s); MK, midkine; Nox, NADPH oxidase; PTN, pleiotrophin; RAS, renin-angiotensin system; rh-MK, recombinant human MK.

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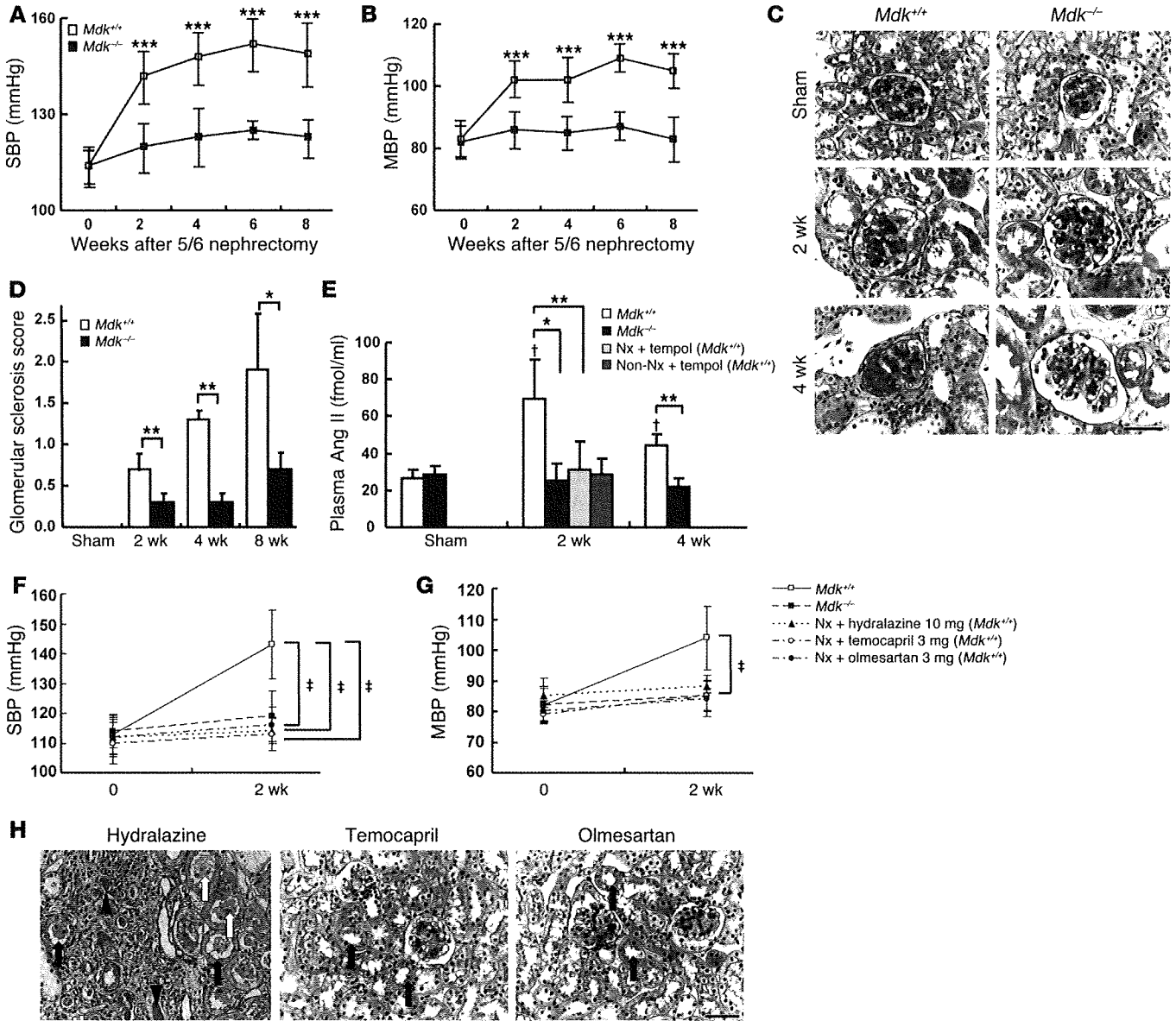

Figure 1

5/6 nephrectomy induces hypertension and renal damage via the RAS. (**A** and **B**) Blood pressure was measured at 0, 2, 4, 6, and 8 weeks after 5/6 nephrectomy. Systolic blood pressure (SBP) (**A**) and mean blood pressure (MBP) (**B**) were measured by the tail-cuff method. The mean and SD are represented by squares and bars, respectively, at each time point ($Mdk^{+/+}$: 0 weeks, $n = 40$; 2 weeks, $n = 34$; 4 weeks, $n = 19$; 8 weeks, $n = 8$; $Mdk^{-/-}$: 0 weeks, $n = 26$; 2 weeks, $n = 23$; 4 weeks, $n = 13$; 8 weeks, $n = 4$). $^{***}P < 0.001$ versus $Mdk^{-/-}$ mice. (**C**) Representative glomerular histology shown by PAS staining. Scale bar: 50 μm. (**D**) Semiquantitative analysis of the glomerular sclerosis score. Data are shown as mean and SD ($Mdk^{+/+}$: 2 weeks, $n = 5$; 4 weeks, $n = 4$; 8 weeks, $n = 4$; $Mdk^{-/-}$: 2 weeks, $n = 4$; 4 weeks, $n = 3$; 8 weeks, $n = 3$). (**E**) Plasma Ang II concentration after 5/6 nephrectomy ($Mdk^{+/+}$: sham, $n = 6$; 2 weeks, $n = 6$; 4 weeks, $n = 8$; $Mdk^{-/-}$: sham, $n = 6$; 2 weeks, $n = 7$; 4 weeks, $n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\dagger}P < 0.01$ versus sham $Mdk^{+/+}$. Nx, nephrectomy. (**F** and **G**) Effects of hydralazine, temocapril, and olmesartan on blood pressure. SBP (**F**) and MBP (**G**) were measured by the tail-cuff method ($n = 3$). $^{\ddagger}P < 0.01$ versus $Mdk^{+/+}$ mice. (**H**) Representative histology after treatment with hydralazine, temocapril, and olmesartan. The kidney specimens were stained with PAS. Tubular dilatation (black arrows), tubular cast formation (arrowheads), and tubular degeneration (white arrows) are indicated. Scale bar: 50 μm.

significant increase (systolic blood pressure, 143 ± 11.6 mmHg in $Mdk^{+/+}$ mice vs. 119 ± 8.6 mmHg in $Mdk^{-/-}$ mice; mean blood pressure, 104 ± 10.3 mmHg vs. 85 ± 6.7 mmHg). Consequently, systolic and mean blood pressures were significantly higher in $Mdk^{+/+}$ than in $Mdk^{-/-}$ mice from 2 to 8 weeks (Figure 1, A and B).

5/6 nephrectomy caused not only hypertension but also progressive renal failure. Blood urea nitrogen and serum creatinine levels gradually increased, and both parameters were significantly higher in $Mdk^{+/+}$ mice at 2 and 4 weeks after renal ablation (Table 1). $Mdk^{+/+}$ mice also exhibited more severe glomerular sclerosis, which is characterized by a marked deposition of extracellular matrix in the glomeruli and which occurred as early as 2 weeks after renal ablation (Figure 1C). Semiquantitative analysis of the glomerular sclerosis scores revealed significant differences between $Mdk^{+/+}$

Table 1
Body weight, blood urea nitrogen, serum creatinine, and left kidney weight after 5/6 nephrectomy

	BW (g)	BUN (mg/dl)	Cre (mg/dl)	Left kidney wt (mg)
Before nephrectomy				
Mdk$^{+/+}$	22.2 ± 2.1	21.2 ± 3.7	0.06 ± 0.02	–
Mdk$^{-/-}$	21.3 ± 1.3	19.7 ± 1.4	0.04 ± 0.02	–
Hydralazine, 10 mg/kg/d	23.1 ± 0.6	ND	ND	–
Temocapril, 3 mg/kg/d	22.0 ± 2.0	ND	ND	–
Olmesartan, 3 mg/kg/d	24.5 ± 0.5	ND	ND	–
Tempol, 3 mmol/l	22.7 ± 1.7	ND	ND	–
2 weeks after nephrectomy				
Mdk$^{+/+}$	19.7 ± 2.2	57.1 ± 9.7	0.65 ± 0.11	104.8 ± 9.6
Mdk$^{-/-}$	18.8 ± 1.5	39.5 ± 7.2^{A}	0.39 ± 0.08^{A}	104.3 ± 15.1
Hydralazine, 10 mg/kg/d	18.7 ± 0.7	63.3 ± 7.6	0.53 ± 0.12	ND
Temocapril, 3 mg/kg/d	19.7 ± 2.7	30.3 ± 2.9^{A}	0.21 ± 0.04^{A}	ND
Olmesartan, 3 mg/kg/d	21.3 ± 0.7	36.0 ± 1.0^{B}	0.21 ± 0.01^{A}	ND
Tempol, 3 mmol/l	21.4 ± 1.6	41.2 ± 3.9^{B}	0.25 ± 0.04^{A}	ND
4 weeks after nephrectomy				
Mdk$^{+/+}$	20.9 ± 1.8	66.1 ± 8.7	0.77 ± 0.13	130.0 ± 21.4
Mdk$^{-/-}$	20.6 ± 1.7	46.4 ± 4.8^{A}	0.44 ± 0.15^{B}	130.3 ± 10.7
8 weeks after nephrectomy				
Mdk$^{+/+}$	22.5 ± 1.9	71.9 ± 14.6	1.56 ± 0.34	118.5 ± 14.9
Mdk$^{-/-}$	21.2 ± 2.3	62.9 ± 12.9	1.18 ± 0.29	128.5 ± 6.0

Values are mean ± SD. BUN, blood urea nitrogen; Cre, serum creatinine. ND, no data. Hydralazine, temocapril, olmesartan, and tempol were administered to *Mdk*$^{+/+}$ mice at the indicated doses. $^{A}P < 0.001$ versus *Mdk*$^{+/+}$. $^{B}P < 0.01$ versus *Mdk*$^{+/+}$.

and *Mdk*$^{-/-}$ mice (Figure 1D). In addition, the tubulointerstitial damage was worse in *Mdk*$^{+/+}$ mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI37249DS1). Thus, tubular dilatation, cast formation in the tubular lumen, and tubular epithelial degeneration became apparent at 2 weeks after renal ablation and were more severe in *Mdk*$^{+/+}$ mice (Supplemental Figure 1, A–H). Interstitial fibrosis, as evidenced by collagen deposition revealed by Masson's trichrome staining, was exhibited at 4 weeks and then more diffusely at 8 weeks, and the stained area became expanded in *Mdk*$^{+/+}$ mice (Supplemental Figure 1, I–P). These data collectively indicate that renal damage was more severe in *Mdk*$^{+/+}$ mice than in *Mdk*$^{-/-}$ mice.

These symptoms of hypertension and renal damage were attributable to the RAS, as (a) the hypertension was accompanied by elevated plasma Ang II concentration (Figure 1E); and (b) the ACE inhibitor temocapril and the angiotensin receptor blocker olmesartan reduced both blood pressure and renal tubulointerstitial damage, but the vasodilator hydralazine only ameliorated hypertension (Figure 1, F–H). Abnormal elevation of blood urea nitrogen and serum creatinine was ameliorated by administration of temocapril and olmesartan, but not hydralazine (Table 1). It is therefore conceivable that the RAS contributed to both hypertension and renal damage.

ACE levels are increased in the lung after 5/6 nephrectomy. Expression of the intrarenal angiotensinogen and renin was suppressed and ACE expression was unchanged in *Mdk*$^{+/+}$ mice, which was consistent with previous findings after 5/6 nephrectomy of rats (19, 20) (Supplemental Figure 2, A–C). In contrast to the kidney, the lung showed significant increases in ACE expression and its activity 2 and 4 weeks after renal ablation (Figure 2, A–E). ACE protein expression was localized to the pulmonary vascular endothelial cells and alveolar-capillary endothelial cells, consistent with a previous report (Figure 2D) (21).

Other organs that play major functions in the RAS were also examined for their expression of RAS components. Angiotensinogen mRNA expression was induced in the liver after 5/6 nephrectomy, but the expression level was not significantly different in *Mdk*$^{+/+}$ and *Mdk*$^{-/-}$ mice (Supplemental Figure 3A). There was no difference in angiotensinogen protein levels between the two genotypes (Supplemental Figure 3, B and C), and renin mRNA expression was not detected in the liver (data not shown). Expression of ACE protein and MK protein was also not detected in the liver (Supplemental Figure 3, D and E). In the brain, mRNA expression of angiotensinogen and ACE did not change after 5/6 nephrectomy (Supplemental Figure 4, A and B), and the renin mRNA expression was undetectable (data not shown). ACE protein was also not detected in the brain (Supplemental Figure 4C). Furthermore, the mRNA expression of angiotensinogen, renin, and ACE did not change in the heart (Supplemental Figure 5). Therefore, it is most likely that the hypertension observed after 5/6 nephrectomy was due to activation of the lung ACE.

MK levels are increased in the lung, kidney, and plasma after 5/6 nephrectomy. MK expression was increased in the lung in association with an elevation in ACE expression (Figure 3, A–C). MK protein was localized to the endothelium of microvessels of the lung, as revealed by the use of thrombomodulin as a marker of the vascular endothelium (Figure 3D). MK expression was detected in alveolar-capillary endothelial cells but not in bronchial epithelial cells (Figure 3D).

Histological evidence of lung damage, i.e., due to edema and degeneration of alveolar cells, was not observed after 5/6 nephrectomy in the *Mdk*$^{+/+}$ and *Mdk*$^{-/-}$ mice (Supplemental Figure 6A). Increases in macrophage and neutrophil infiltration into the lung were also not observed after 5/6 nephrectomy in the two genotypes (Supplemental Figure 6, B–E). These results indicated that the increase in MK expression in the lung after 5/6 nephrectomy was not due to leukocytes.

MK expression was also significantly elevated in the kidney at both the protein and mRNA levels 2 and 4 weeks after renal ablation (Supplemental Figure 7, A–C). Immunohistochemical analysis revealed that MK protein was mainly localized in the tubular epithelium (Supplemental Figure 8A). This result is consistent with previous reports in which MK was expressed in the kidney after ischemia/reperfusion injury and its associated massive leukocyte infiltration (22). We also detected a substantial increase in macrophage infiltration into the kidney after 5/6 nephrectomy, and this increase was significantly higher in *Mdk*$^{+/+}$ mice than *Mdk*$^{-/-}$ mice (Supplemental Figure 8, B and C). It is known that MK is expressed by activated macrophages (23, 24). Thus, it is conceivable that the increase in MK expression in the kidney after 5/6 nephrectomy was due to enhanced expression in both the tubular epithelium and infiltrating macrophages.

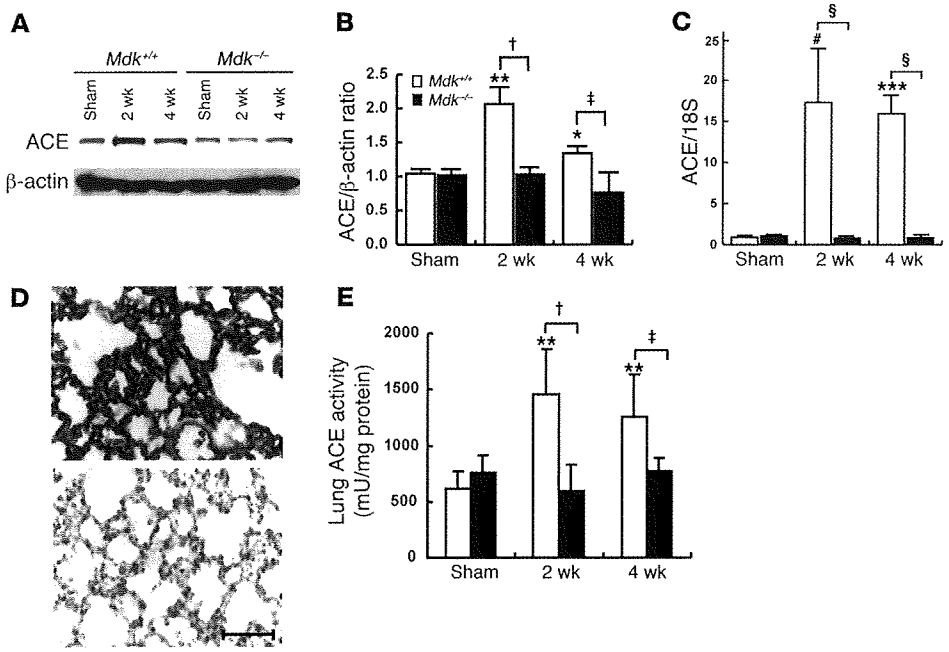

Figure 2

ACE expression in the lung after 5/6 nephrectomy. (**A**) ACE protein was determined by Western blotting, and a representative result is shown. The lung tissues were obtained at the indicated time points. (**B**) Quantitative analysis of ACE protein expression using densitometry. Data are presented as mean and SD ($n = 3$). $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.05$; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham $Mdk^{+/+}$. (**C**) ACE mRNA was determined by real-time PCR and normalized to 18S mRNA. Data are presented as mean and SD ($Mdk^{+/+}$: sham, $n = 5$; 2 weeks, $n = 5$; 4 weeks, $n = 3$; $Mdk^{-/-}$: sham, $n = 3$; 2 weeks, $n = 3$; 4 weeks, $n = 3$). $^{\S}P < 0.0001$; $^{***}P < 0.001$ and $^{\#}P < 0.0001$ versus sham $Mdk^{+/+}$. (**D**) Immunohistochemical staining of lungs with mouse anti-ACE monoclonal antibody at 2 weeks. Upper panel: The first antibody used was anti–mouse ACE monoclonal antibody. Lower panel: Isotype-matched IgG was used as the first antibody. Scale bar: 50 μm. (**E**) ACE activity was determined by the ACE activity assay, as described in Methods. Data are presented as mean and SD ($Mdk^{+/+}$: sham, $n = 5$; 2 weeks, $n = 5$; 4 weeks, $n = 5$; $Mdk^{-/-}$: sham, $n = 5$; 2 weeks, $n = 5$; 4 weeks, $n = 4$). $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.05$; $^{**}P < 0.01$ versus sham $Mdk^{+/+}$.

Since MK is a secreted protein, and its expression was induced by 5/6 nephrectomy in the kidney and lung (Figure 3, A–C, and Supplemental Figure 7, A–C), we next examined plasma MK levels. As shown in Figure 3, E and F, plasma MK levels were indeed increased 2 weeks after 5/6 nephrectomy.

Exogenous MK induces ACE expression. If MK is required for ACE expression in the lung and hypertension, supplementary administration of MK might also affect these symptoms. To clarify this issue, we administered exogenous MK continuously through an osmotic pump into $Mdk^{-/-}$ mice after 5/6 nephrectomy. This administration was found to restore hypertension and ACE expression in the lung (Figure 4, A–C). We also administered pleiotrophin (PTN; also called HB-GAM), which shows 50% homology with MK (8), to $Mdk^{-/-}$ mice after 5/6 nephrectomy. However, exogenous PTN neither induced hypertension nor increased ACE expression in the lung (Figure 4, A, D, and E). These data support the specificity of MK with respect to its involvement in ACE expression and blood pressure regulation.

Furthermore, exogenous MK protein on primary cultured human lung microvascular endothelial cells (HMVEC-L) significantly enhanced ACE expression, suggesting that ACE is one of the targets of MK in the lung (Figure 5, A and B). When Ang I was added to the culture medium of the lung endothelial cells treated with MK and heparin for 36 hours, Ang I was converted to Ang II in a time-dependent manner, while cells treated with heparin alone did not show such a conversion (Figure 5C). These results suggest that MK is a potent inducer of Ang II through upregulation of ACE expression in lung endothelial cells.

Along with the increase in ACE expression, phosphorylation levels of PKC were also increased in primary cultured HMVEC-L after exposure to exogenous MK (Figure 5, D and E). This result suggests that MK upregulates ACE expression through activation of PKC. This idea was further supported by three lines of evidence. First, bisindolylmaleimide I (BIS), a PKC-specific inhibitor, blocked the MK-mediated increase in ACE expression (Figure 5, A and B). Second, PKC phosphorylation was significantly increased in the lungs of $Mdk^{+/+}$ but not $Mdk^{-/-}$ mice after 5/6 nephrectomy (Figure 5F). Third, consistent with previous reports (25), the increase in ACE expression in primary cultured HMVEC-L was also induced by PMA, a PKC activator, and was blocked by BIS (Supplemental Figure 9).

Oxidant stress induces MK expression in the lung after 5/6 nephrectomy. Finally, the mechanism of MK induction in the lung by 5/6 nephrectomy was investigated. The NADPH oxidases (Nox's) are superoxide-generating enzymes that release superoxide by electron transfer from NADPH to oxygen. Increased production of ROS has been implicated in various pathologies, including hypertension, atherosclerosis, diabetes, and CKD (26, 27). In the present study, Nox1, -2, and -4 mRNA expression in the lungs of $Mdk^{+/+}$ mice was found to be significantly increased at 2 and 4 weeks after renal ablation compared with the levels in the sham-operated animals, suggesting that oxidant stress was generated in the lung; in contrast, the expression of Nox1, -2, and -4 mRNA was unchanged in the $Mdk^{-/-}$ mice (Figure 6A). A cell membrane–permeable radical scavenger, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (tempol), reduced MK expression to normal levels in the lung (Figure 6B); plasma Ang II levels (Figure 1E) and blood pressure (Figure 6C) were also reduced. Tempol also ameliorated glomerular sclerosis and tubulointerstitial damage (Figure 6D) and improved renal function, i.e., significantly reduced blood urea nitrogen and serum creatinine levels (Table 1). These results suggest that MK expression was induced by oxidative stress in the lung after 5/6 nephrectomy. Tempol also reduced MK expression in the kidney (Supplemental Figure 7, D and E).

Discussion

Our study demonstrated that $Mdk^{-/-}$ mice had almost normal blood pressure after 5/6 nephrectomy, while wild-type mice showed

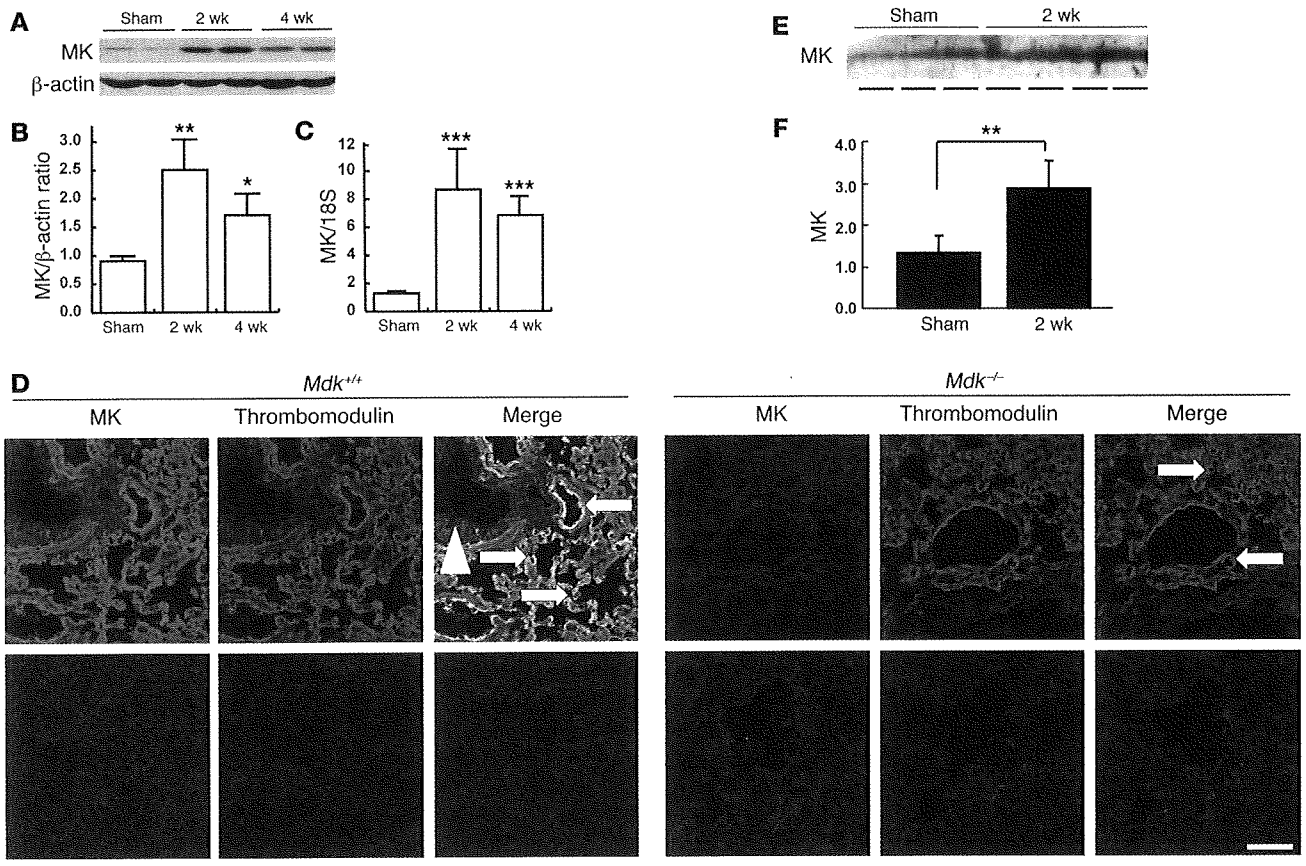

Figure 3

MK expression in the lung and plasma after 5/6 nephrectomy. (**A**) Representative data from Western blotting for MK expression in the lung. (**B**) The intensity of MK bands on Western blotting was normalized to that of β-actin. Data are presented as mean and SD ($n = 4$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham. (**C**) MK mRNA expression in the lung was determined by real-time PCR and normalized to 18S mRNA. Data are presented as mean and SD (sham, $n = 5$; 2 weeks, $n = 5$; 4 weeks, $n = 3$). $^{***}P < 0.001$ versus sham. (**D**) Immunofluorescence staining of MK and thrombomodulin expression in the lung 2 weeks after 5/6 nephrectomy. Lower panels show negative controls using isotype-matched IgG as the first antibody. Arrowhead, bronchial epithelial cells; white arrows, alveolar-capillary endothelial cells. Scale bar: 50 μm. (**E**) Representative data from Western blotting for MK expression in plasma are shown. Lines under the blot indicate individual samples. (**F**) Western blot data for plasma MK were quantified using densitometry and are presented as mean and SD (sham, $n = 4$; 2 weeks, $n = 5$). $^{**}P < 0.01$.

marked hypertension. This hypertension was ameliorated by RAS-related inhibitors and indeed was accompanied by elevated plasma Ang II levels. Surprisingly, ACE activity was enhanced in the lung, whereas RAS components were not activated in other organs. Plasma MK levels and MK expression in the lung and kidney were elevated. Supplementary MK administration to *Mdk*$^{-/-}$ mice restored lung ACE expression and hypertension. MK also induced ACE expression and consequently conversion from Ang I to Ang II in primary cultured lung microvascular endothelial cells. We therefore concluded that MK-mediated ACE induction in the lung is critical for hypertension induced by 5/6 nephrectomy (Figure 7).

Inter-organ interactions involving the kidney have recently been highlighted. Regarding factors affecting lung function after acute kidney injury, several cytokines, including IL-6, IL-1β, and TNF-α, have been suggested as candidates (28, 29). Such results contribute to our understanding of the high mortality associated with pulmonary complications following acute kidney injury. CKD has also been linked with damage in other organs, especially with cardiovascular damage (so-called cardiorenal syndrome) (30). It is particularly interesting that RAS components are increased in the heart and brain of subtotally nephrectomized rats (20, 31) and that Ang II amounts are increased in the isolated perfused hind limbs of uremic rats (32). Inhibitors of the RAS, e.g., angiotensin receptor blockers and ACE inhibitors, are indeed the first choice of therapy for CKD (33). Our results clearly show that the lung is a promising target in the cross-talk between the kidney and other organs and suggest that MK is a candidate mediator for pulmonary and other organ complications associated with CKD.

Regarding the cross-talk between the kidney and lung in 5/6 nephrectomy, our study has also provided an insight into the underlying mechanism. We found that Nox1, -2, and -4 were induced in the lung and that tempol reduced MK and plasma Ang II levels. Nox mediates the initial reaction of 3 successive reduction products of molecular oxygen, i.e., superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). Since tempol is a membrane-permeable and metal-independent superoxide dismutase mimetic that is specific for superoxide anion ($O_2^{\bullet -}$) (34, 35), tempol may target ROS initiated by Nox in the lung. To the best of our knowledge, this is the first study to show that 5/6 nephrectomy induces oxidative stress in the lung. We have previously reported that oxidative