

Nitric oxide donors rescue diabetic nephropathy through oxidative-stress- and nitrosative-stress-mediated Wnt signaling pathways

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Keywords

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ABSTRACT

Aims/Introduction: The role of the renal nitric oxide (NO) system in the pathophysiology of diabetic nephropathy constitutes a very challenging and fertile field for future investigation. The purpose of the present study was to investigate whether NO donors can attenuate diabetic renal fibrosis and apoptosis through modulating oxidative- and nitrosative-stress, and Wnt signaling using *in vivo* diabetic models.

Materials and Methods: Diabetic rat was induced by a single intraperitoneal injection of streptozotocin. Rats in each group were intraperitoneally given 2,2'-(hydroxynitrosohydrazino)bis-ethanamine (1 U/kg/day) and vehicle for 28 and 56 consecutive days. Expression of the oxidative- and nitrosative-stress, and Wnt signaling components were examined in kidneys from diabetic animals by quantitative reverse transcription polymerase chain reaction, western blot analysis and immunohistochemical staining.

Results: NO donor treatment significantly reduced the ratio of kidney weight to body-weight and proteinuria. This treatment also significantly restored the suppressive effect of diabetes on urinary NO₂ + NO₃ levels. Immunohistochemistry showed that NO donor treatment significantly reduced transforming growth factor (TGF)-β1, fibronectin, cleaved caspase-3 and triphosphate-biotin nick end-labeling expression in the glomeruli of diabetic rats. We found that diabetes promoted 8-hydroxy-2'-deoxyguanosine, and peroxynitrite expression coincided with reduced endothelial NO synthase expression in glomeruli. Interestingly, NO donor treatment completely removed oxidative stress and nitrosative stress, and restored endothelial NO synthase expression in diabetic renal glomeruli. Immunohistomorphometry results showed that NO donor treatment significantly restored suppressed Wnt5a expression and β-catenin immunoreactivities in glomeruli. Based on laser-captured microdissection for quantitative reverse transcription polymerase chain reaction, diabetes significantly increased TGF-β1, and fibronectin expression coincided with depressed Wnt5a expression. NO donor treatment reduced TGF-β1, fibronectin activation, and the suppressing effect of diabetes on Wnt5a and β-catenin expression in renal glomeruli.

Conclusions: NO donor treatment alleviates extracellular matrix accumulation and apoptosis in diabetic nephropathy *in vivo* by not only preventing the diabetes-mediated oxidative and nitrosative stress, but also restoring downregulation of endothelial NO synthase expression and Wnt/β-catenin signaling. These findings suggest that modulation of NO is a viable alternative strategy for rescuing diabetic renal injury.

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INTRODUCTION

Diabetic nephropathy is a leading cause of end-stage renal disease (ESRD), accounting for 35–40% of all new ESRD cases that require dialysis therapy worldwide¹. Although current medical care has been improved, the number of diabetic patients with nephropathy progression to ESRD is still increasing. In comparison with other types of chronic renal disease, diabetic nephropathy is associated with rapid functional deterioration, and eventually progresses to renal failure requiring hemodialysis¹. Diabetic nephropathy is distinguished histologically by glomerular hypertrophy, thickening of the glomerular basement membrane, expansion of the mesangial matrix and final glomerulosclerosis^{2,3}. Marked and progressive accumulation of the mesangial matrix, as a result of increased synthesis and decreased degradation of mesangial extracellular matrix proteins that include collagen IV and fibronectin^{4–6}, ultimately obliterates the glomerular capillary loops and leads to renal failure.

Oxidative stress and nitrostatic stress mediates the deleterious effects of diabetes on renal tissue function. The high glucose induction of reactive oxygen radicals both interrupts microvasculature and promotes collagen IV accumulation in mesangial cells^{7–9}. The suppression of oxidative stress by modulating oxidase activity and anti-oxidant capacity alleviates high glucose-mediated mesangial fibrosis^{10,11} and renal cell apoptosis^{12,13}. In an earlier study, we showed that the Ras induction of oxidative stress mediated fibrosis matrix accumulation in the early stage of high glucose-induced renal injuries¹⁴. Furthermore, we have also shown that Wnt/ β -catenin signaling is involved in the regulation of redox signaling in diabetic nephropathy¹⁵. In other words, we have clearly shown that the Wnt/ β -catenin signaling pathway is of fundamental importance in the search for a strategy to rescue diabetic renal injury. Specifically, manipulation of the Wnt/ β -catenin pathway could rescue high glucose-induced fibrosis and apoptosis in diabetic nephropathy^{16,17}.

Nitric oxide (NO) is produced from a guanidino-nitrogen of L-arginine and dioxygen by NO synthases. In recent years, NO has become established as a diffusible universal messenger mediating cell–cell communication throughout the body. The interaction of NO with molecular oxygen (O_2) or O_2^- gives rise to the formation of the potent nitrostatic agents, N_2O_3 and peroxynitrite ($ONOO^-$), respectively. For endothelial NO synthase knockout mice (eNOS KO), it has been reported that a lack of eNOS accelerates both glomerular and tubulointerstitial injury with a loss of glomerular capillaries and peritubular capillaries. Impaired endothelial function is likely a direct risk factor for renal disease¹⁸. In addition, eNOS KO mice have been reported to develop focal congenital renal abnormalities, including glomerular hypoplasia and tubular cell death, and atubular glomeruli¹⁹. Furthermore, recent studies have shown that eNOS KO mice with advanced diabetic nephropathy receive less benefit from renin–angiotensin blockade than from aldosterone receptor antagonist²⁰. However, the literature pertaining to the

role of NO abnormalities in diabetic nephropathy is not only vast, but also composed of confusing and conflicting data. A diabetic metabolic milieu can trigger a variety of autocrine and paracrine mechanisms that modulate the renal NO system, with both stimulatory and inhibitory signals acting in parallel. As a result, the role of the renal NO system in the pathophysiology of diabetic nephropathy remains a complex area with conflicting observations, and constitutes a very challenging and open field for future investigation. We thus hypothesize that modulation of NO can attenuate diabetic nephropathy by modulating both oxidative stress and renal injury from Wnt signaling mediation.

The purposes of the present study were to investigate whether NO donors can attenuate diabetic renal fibrosis and apoptosis through the modulation of oxidative stress and nitrostatic stress in diabetic glomerular injury, and whether NO donors regulate Wnt signaling in the kidney microenvironment in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Streptozotocin-Induced Diabetes

All studies were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital, Taiwan. Four-month-old male Wistar rats (National Experimental Animals Production Center, Taipei, Taiwan) were caged in pairs, and maintained on rodent chow and water ad libitum. Rats with diabetes were induced according to previously described procedures¹⁴. Briefly, diabetes was induced by a single intraperitoneal injection of streptozotocin (100 mg/kg bodyweight; Sigma Chemical Inc., St Louis, MO, USA). Streptozotocin was dissolved in 50 mmol/L citric acid and sterile filtered through a 0.22-mm filter. One week after injection, blood sugar was measured by tail bleeding. Rats with serum glucose >300 mg/dL were used for the following experiments.

NO Donor (NOC-18) Treatment

To understand the effects of NO donors on normal rats, six normal control rats were randomly divided into two groups. Rats ($n = 3$) in each group were intraperitoneally given 2,2'-(hydroxynitrosohydrazino)bis-ethanamine (NOC-18; 1 U/kg/day) and vehicle for 28 and 56 consecutive days, respectively. A total of 16 rats with diabetes were randomly divided into two groups. Rats ($n = 8$) in each group were intraperitoneally given NOC-18 (1 U/kg/day) and vehicle for 28 and 56 consecutive days, respectively. Eight rats without streptozotocin injection were used as the normal control. At day 28 and 56, blood was harvested from each group of rats by intracardiac needle and processed to collect the mononuclear cell fraction using Ficoll-Paque separation as previously described²¹ and for measuring levels of glycated hemoglobin (HbA1c) according to the international standard^{22,23}. Urine total protein and creatinine levels were determined using respective assay kits (Sigma Chemical Inc., St. Louis, MO, USA) according to the manufacturer's

instructions. Rats were killed using an overdose of pentobarbital sodium and kidneys were dissected for immunohistochemistry.

Measurement of NO

We measured nitrite and nitrate levels in serum and urine to reflect NO production as previously described. The serums were obtained in triplicate by injecting of 50 μ L aliquot into a custom imprinter with a Teflon-linked septum containing 0.4 mol/L vanadium chloride in glacial acetic acid. The vanadium chloride reduced nitrate and nitrite to NO gas, which passed into a stream of helium and entered the nitrogen oxide analyzer (NOA280; Sievers Inc., Denver, CO, USA)²⁴. The nitrite and nitrate level in each sample was determined by interpolation calculated from a series of well-known potassium nitrate concentrations. Results were normalized with protein concentration in each sample using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Kidneys were fixed in 4% phosphate-buffered saline (PBS)-buffered paraformaldehyde, decalcified, embedded in paraffin and then sliced longitudinally into 5- μ m thick sections. For immunohistochemistry, the following reagents were used: monoclonal antibodies against, Wnt5a and β -catenin, (Cell Signaling Technology Inc., Beverly, MA, USA), transforming growth factor (TGF)- β 1, fibronectin, 8-hydroxy-guandine, cleaved caspase-3 and eNOS (Chemicon International Inc., Temecula, CA, USA). ONOO⁻ (peroxynitrite) was assessed by nitrotyrosine immunohistochemistry (Chemicon International Inc.). Immunoreactivity in sections was shown using a horseradish peroxidase-3', 3'-diaminobenzidine (DAB) kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions, followed by counterstaining with hematoxylin, dehydration and mounting. Sections without primary antibodies were enrolled as negative controls for the immunostaining.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End-Labeling

Apoptotic cells in cell cultures and kidney tissue were detected using *in situ* cell death detection kits (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions as previously described¹⁵.

Kidney Tissue Preparation and Microdissection

Kidneys were dissected and weighed. After perfusion with PBS, fresh kidney tissues were fixed in 4% PBS-buffered formaldehyde and paraffin embedded under a ribonuclease-free condition. Specimens were sliced longitudinally into 4- μ m thick sections and transferred onto poly-lysine-coated slides. Fresh kidney tissues were also ground with a mortar and pestle under liquid nitrogen in a ribonuclease-free condition to harvest total ribonucleic acid (RNA) for quantitative reverse transcription polymerase chain reaction (RT-PCR) assessment. In some experiments, glomerular mesangium in the formaldehyde-fixed

renal sections were harvested by a laser capture microdissector (VERITASTM; Arcturus Bioscience Inc., Mountain View, CA, USA) according to the manufacturer's instructions. A total of 200 glomerular mesangium from six sections of each rat in each group were dissected in order to extract total RNA and quantitative RT-PCR¹⁷.

Real-Time PCR

Total RNA was extracted from 106 glomerular mesangium cells using Tri reagent (Sigma Chemical Inc., St. Louis, MO, USA). Then, 1 μ g of total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA). A total of 25 μ L of PCR mixture containing cDNA template (equivalent to 20 ng total RNA), 2.5 nmol/L each of forward primer, reverse primer and 2X iQTM SYBR Green Supermix, was amplified by using an iCycler iQ[®] Real-time PCR Detection System (Bio-Rad Laboratories) with an initial melt at 95°C for 5 min followed by 40 cycles at 94°C for 15 s, 52°C for 20 s and 72°C for 30 s. The following gene-specific primers were used: TGF- β 1 (forward: 5'-TGA GTG GCT GTC TTT TGA CG-3'; reverse: 5'-TGG GAC TGA TCC CAT TGA TT-3'); fibronectin (forward: 5'-GTG GCT GCC TTC AAC TTC TC-3'; reverse: 5'-AGT CCT TTA GGG CGG TCA AT-3'); Wnt5a (forward: 5'-AGC CGA GAG ACA GCC TTC AC -3'; reverse: 5'-TCC TGC GAC CTG CTTTCATTG-3'; 289 bp expected); the β -catenin gene (forward: 5'-ACAAACTGTTTGTAAAATCCA-3'; reverse: 5'-CGAGT-CATTGCATACTGTCC-3') and β -actin (forward: 5'-CGC CAA CCG CGA GAA GAT-3'; reverse: 5'-CGT CAC CGG AGT CCA TCA-3'). The number of amplification steps required to reach an arbitrary intensity threshold (C_t) was computed. The relative gene expression was represented as $2^{-\Delta C_t}$, where $\Delta C_t = C_{t\text{target}} - C_{t\beta\text{-actin}}$. Fold change for the treatment was calculated as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_{t\text{treatment}} - \Delta C_{t\text{vehicle}}$.

Histomorphometry

Three random images of 0.75 mm² from each area (3 mm²) were then taken under $\times 400$ magnifications using a Cool CCD camera (SNAP-Pro cf Digital kit; Media Cybernetics, Silver Spring, MD, USA). Five sections of the glomerular area of each rat were collected and each section was then divided into five sub-areas. To semi quantify the number of positive immune-labeled cells in the glomeruli, the integral optical density (IOD) was analyzed using Image-Pro[®] Plus version 6.0 image-analysis software (Media Cybernetics, Silver Spring, MD, USA). For each rat, we presented the average of 25 sub-areas.

Western Blotting

Renal tissue extracts were isolated and subjected to electrophoresis, blotted and probed by antibodies against phosphorylated eNOS at Ser¹¹⁷⁷, eNOS (Cell Signaling Technology Inc.) and dephospho- β -catenin (Upstate Biotechnology, Inc., Lake Placid, NY, USA), followed by probing with horseradish peroxidase conjugated immunoglobulin G and visualization with chemiluminescence reagents.

Statistical Analysis

All values were expressed as the mean ± standard error. The Wilcoxon test was used to evaluate differences between the sample of interest and its respective control. For analysis of concentration effect and time course, a multiple range of anova and Bonferroni post-hoc tests were used. *P* < 0.05 was considered significant.

RESULTS

Exogenous NO Donors for 8 Weeks But Not 4 Weeks Alleviated Urinary Total Proteinuria Secretion of Kidney in Diabetic Rats

We examined whether *in vivo* donation of NO could alter diabetes induction of glomerulopathy. Diabetic rats were given NOC-18 for 28 and 56 days. There was no significant difference in blood glucose, HbA1c, ratio of kidney weight-to-bodyweight, and total urinary excretion in 4 weeks and 8 weeks (Supporting Information). In comparison with the normal group, diabetes significantly increased blood glucose, HbA1c, ratio of kidney weight-to-bodyweight, and total urinary excretion in 4 and 8 weeks. NOC-18 treatment for 4 and 8 weeks did not evidently alter blood glucose and HbA1c throughout the study period. Interestingly, both 4- and 8-week NO donor treatment significantly reduced the ratio of kidney weight-to-bodyweight compared with that of the diabetic group. As for total urinary protein to creatinine ratio (mg/mg), NOC-18 treatment for 4 weeks marginally, but not significantly, reduced the promoting effect of diabetes on urinary protein secretion, whereas NOC-18 treatment significantly reduced the promoting effect of diabetes on urinary protein secretion (Table 1).

Table 1 | Biochemical properties in normal rats, diabetic rats and diabetic rats with NOC-18 treatment

	Normal	DM	
		Vehicle	NOC
4 weeks			
Blood glucose ¹	65.7 ± 1.0	282.8 ± 22.3*	256.5 ± 30.9
Bodyweight (g) ²	403.60 ± 10.87	363.71 ± 13.13*	382.14 ± 18.05
Kidney/BW (%) ²	0.63 ± 0.01	1.18 ± 0.07*	0.99 ± 0.07 [#]
Urine (TP/Cr) ¹	0.74 ± 0.2	2.66 ± 0.3*	2.14 ± 0.3
8 weeks			
Blood glucose ¹	66.1 ± 0.6	262.5 ± 11.4*	236.2 ± 22.6
Bodyweight(g) ²	392.33 ± 9.02	379.25 ± 19.10	388.67 ± 11.77
Kidney/BW(%) ²	0.633 ± 0.003	1.273 ± 0.110*	0.951 ± 0.035 [#]
Urine (TP/Cr) ¹	0.78 ± 0.3	2.90 ± 0.7	1.36 ± 0.1 [#]

¹Data are means ± standard errors calculated from eight rats. Blood glucose (mg/dL) was measured from tails. Total urinary protein secretion (TP/Cr; mg/mg creatinine) was assayed using urine protein kits and normalized with total creatinine level in urine. ²Data are mean ± standard errors calculated from eight rats. *[#]Difference between the normal and diabetes (DM) vehicle groups, DM vehicle and nitric oxide donor (NOC) treatment groups, respectively (*P* < 0.05). BM, bodyweight.

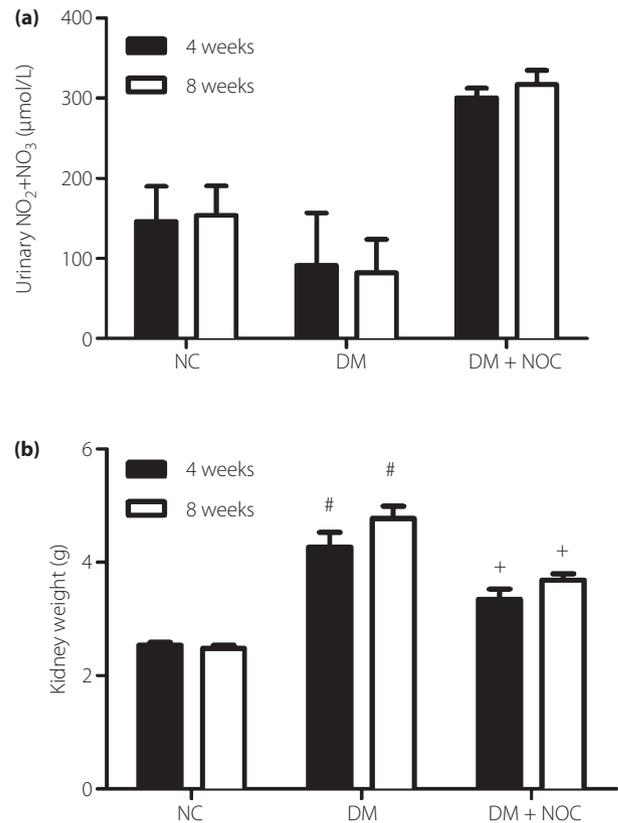


Figure 1 | Exogenous 2,2'-(hydroxynitrosohydrazino)bis-ethanamine (NOC; 1 U/kg/day) treatments (a) significantly restored the suppressive effect of diabetes on urinary NO₂ + NO₃ levels and (b) downregulated enlarged diabetic kidneys. Data are expressed as mean ± standard error. [#]*P* < 0.05 vs control group (NC), ⁺*P* < 0.05 diabetes (DM) group versus nitric oxide (NO) donor treatment group.

NO Donors Restored Urinary NO Levels in 4-Week and 8-Week Diabetic Rats

In comparison with the normal control group, diabetes significantly decreased urinary NO₂ + NO₃ levels and coincided with enlarged diabetic kidneys. Exogenous NOC-18 (1 U/kg/day) treatments significantly restored the suppressive effect of diabetes on urinary NO₂ + NO₃ levels and downregulated enlarged diabetic kidneys (Figure 1).

Exogenous NO Donors Alleviated TGF-β1, Fibronectin and Triphosphate-Biotin Nick End-Labeling Expression of the Kidney in Diabetic Rats

In the absence of primary antibodies, there was no immunostaining of either TGF-β1 or fibronectin. Mesangial cells in glomeruli showing positive for immuno-expression exhibited a brown color in cell periphery and cytoplasm. In the control group, cells located at the glomeruli in the renal cortex expressed weak fibronectin and TGF-β1 expression. In the diabetes group, in contrast, mesangial cells showed strong fibronectin and TGF-β1 expression. Mesangial cells expressed slight

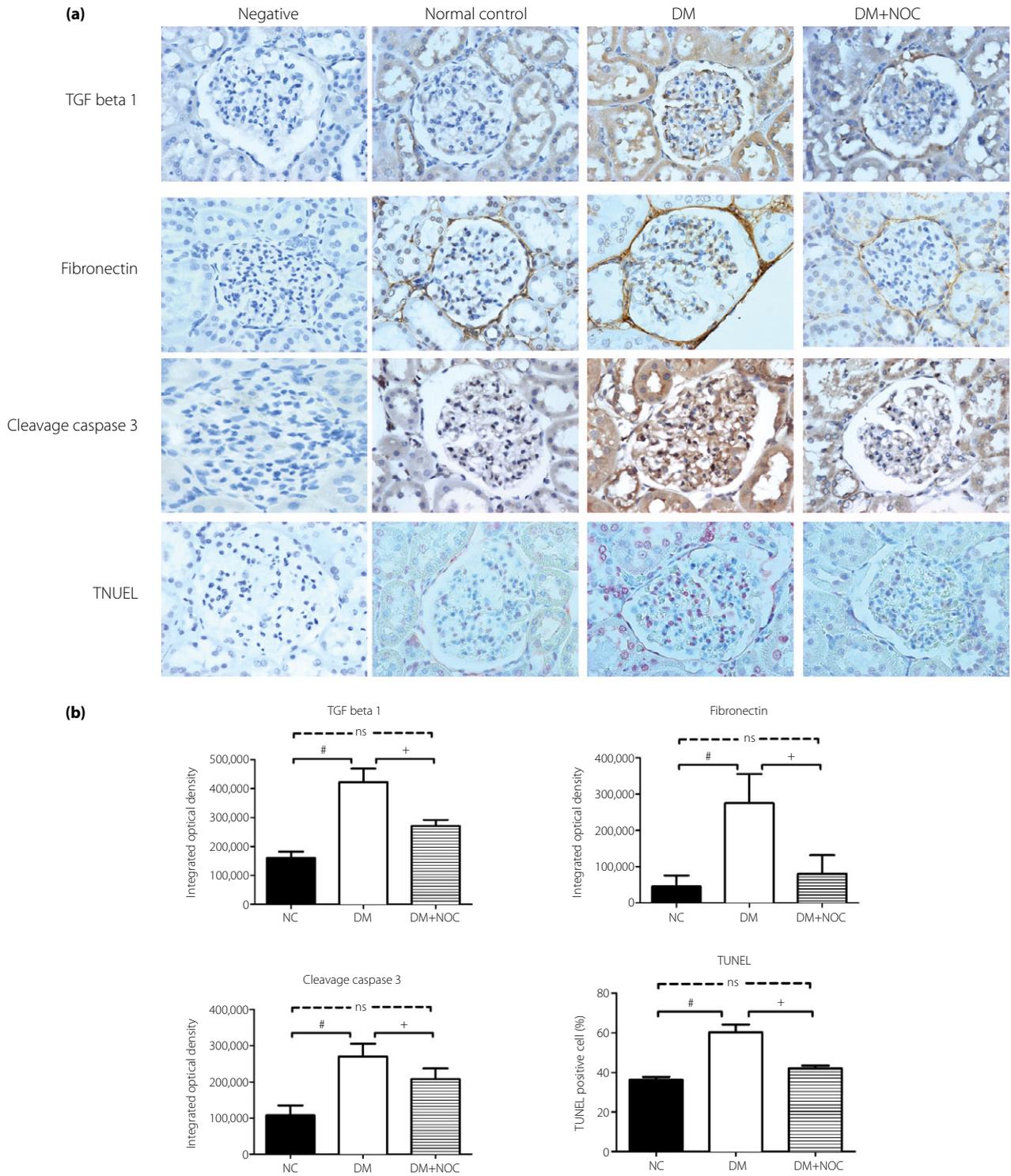


Figure 2 | (a) Immunohistochemical photograph of glomeruli in diabetic rats (DM) with or without nitric oxide (NO) donor (NOC) treatment. In comparison with the control group (NC), mesangial cells at glomeruli in diabetic kidneys expressed stronger transforming growth factor (TGF)- β 1, fibronectin, cleaved caspase-3 and positive triphosphate-biotin nick end-labeling (TUNEL) immunostaining. Decreased TGF- β 1, fibronectin, cleaved caspase-3 and TUNEL expression were observed in diabetic rats after NO donor treatment. (b) Semi quantitative evaluation of the positive immune-labeled cells. Data are expressed as mean \pm standard error. # P < 0.05 versus control group, + P < 0.05 vs NO donor treatment group. Specimens were observed under magnification \times 400.

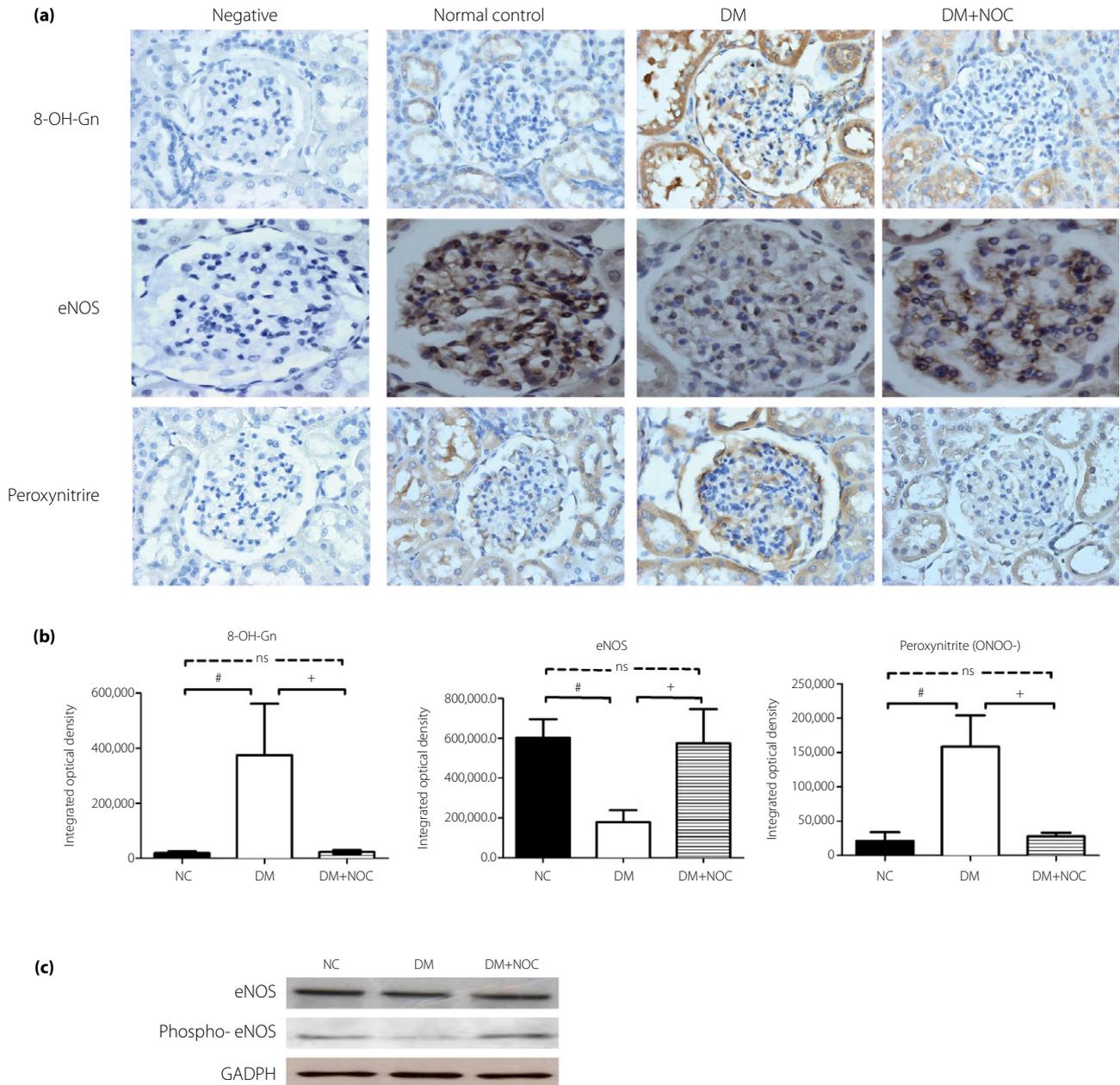


Figure 3 | (a) Immunohistochemical photograph of glomeruli in diabetic rats (DM) with and without nitric oxide (NO) donor (NOC) treatment. In comparison with the control group (NC), mesangial cells at glomeruli in diabetic kidneys expressed stronger 8-hydroxy-2'-deoxyguanosine and peroxynitrite immunostaining. Decreased 8-hydroxy-2'-deoxyguanosine and peroxynitrite expression were observed in diabetic rats after NO donor treatment. The diabetic group (DM) expressed weak endothelial NO synthase expression when compared with the normal group and restored diabetes attenuation of eNOS expression by NO donor treatment. (b) Semi quantitative evaluation of the positive immune-labeled cells. (c) Western blot analysis of homogenized kidney show that phosphorylated (Phospho) eNOS was significantly decreased in the diabetes group compared with the sham group. The restoring effect was noted in the NO donor treatment group in diabetic animals. Data are expressed as mean \pm standard error. # $P < 0.05$ vs control group, * $P < 0.05$ versus NO donor treatment group. Specimens were observed under magnification $\times 400$. GADPH, glyceraldehyde 3-phosphate dehydrogenase.

TGF- β 1 and fibronectin in the diabetic renal cortex after NO donor treatment. In the diabetes group, mesangial cells in the glomeruli showed strong DNA fragmentation, as demonstrated

by positive triphosphate-biotin nick end-labeling (TUNEL) staining. In contrast, few cells in the glomeruli of diabetic rats with NO donor treatment showed weak TUNEL staining

(Figure 2a). Immunohistomorphometry results showed that diabetes significantly increased TGF- β 1, fibronectin, cleaved Caspase-3 and TUNEL expression of glomerular mesangial cells compared with those in the control group. Exogenous NO donor treatment significantly reduced TGF- β 1, fibronectin, cleaved caspase-3 and TUNEL expression in the renal glomeruli of the diabetic rats (Figure 2b). In summary, exogenous NO donor treatment significantly downregulated apoptosis, and coincided with reduced extracellular matrix accumulation in the renal glomeruli of diabetic rats.

Exogenous NO Donors Alleviated Oxidative Stress and Nitrosative Stress Expression of the Kidney in Diabetic Rats

Superoxide easily reacts with nucleotides resulting in DNA damage to form 8-hydroxy-2'-deoxyguanosine (a marker of DNA oxidative damage). Furthermore, the reaction of superoxide with NO produces peroxynitrite, which is a marker of nitrosative stress. We found that diabetes promoted 8-hydroxy-2'-deoxyguanosine and peroxynitrite expression in kidney tissue, whereas NO donor treatment alleviated the diabetes promotion of 8-hydroxy-2'-deoxyguanosine and peroxynitrite expression. Furthermore, in the diabetes group, cells located at glomeruli in the renal cortex expressed weak eNOS expression when compared with those of the normal group. NO donor administration evidently restored diabetes attenuation of eNOS expression (Figure 3a). Immunohistomorphometry results showed that diabetes significantly increased 8-hydroxy-2'-deoxyguanosine and peroxynitrite expression of glomerular mesangial cells compared with those in the control group. Exogenous NO donor treatment significantly upregulated eNOS, and coincided with reduced oxidative and nitrosative stress in the renal glomeruli of diabetic rats (Figure 3b). We carried out western blot analysis to determine eNOS protein expression in diabetic kidney. Diabetic kidney resulted in a significant downregulation of eNOS protein expression. Exogenous NO donor treatment significantly restored eNOS expression in the diabetic treatment group (Figure 3c).

Exogenous NO Donors Restored Wnt Signaling Expression of Kidney in Diabetic Rats

We investigated whether NO donor treatment could alter Wnt5a, and β -catenin expression in diabetic kidneys. In the diabetes group, cells located at the glomeruli in the renal cortex expressed weak Wnt5a and β -catenin expression when compared with the normal group. NO donor administration evidently restored diabetes attenuation of Wnt5a and β -catenin expression (Figure 4a). Immunohistomorphometry results showed that exogenous NO donor treatment significantly suppressed mesangial cell apoptosis, and coincided with restored Wnt5a expression and β -catenin immunoreactivities in the renal glomeruli of diabetic rats (Figure 4b). Immunoblotting showed that diabetes reduced dephosphorylated β -catenin expression. NO donor significantly restored dephosphorylated β -catenin accumulation in diabetic rats (Figure 4c).

NO Donor Treatment Alleviated Extracellular Matrix Accumulation in Renal Mesangial Cells

Glomerular mesangium in the renal tissue were harvested by laser-captured microdissection to extract the total RNA and quantitative RT-PCR (Figure 5). Diabetes significantly increased TGF- β 1 and fibronectin expression of glomerular mesangium. NO donor treatment significantly alleviated diabetes-induced promotion of TGF- β 1 and fibronectin expression of glomerular mesangium. Real-time PCR showed that diabetes reduced Wnt5a and β -catenin messenger RNA expression. NO donor treatment reduced the suppressing effect of diabetes on Wnt5a and β -catenin messenger RNA expression in the diabetic glomeruli (Figure 5).

DISCUSSION

In the present study, it was found that in diabetic animal kidneys high glucose raised oxidative stress and nitrosative stress in association with downregulated Wnt signaling activity, and progressively promoted fibrogenic and apoptotic activity. Although our recent reports show the functional role of Wnt/ β -catenin in responding to high glucose^{14,15,17}, little research has been carried out to define the biological role of NO donors in diabetes-induced nephropathy in diabetic animals. The present findings show that NO donor treatment alleviates extracellular matrix accumulation and apoptosis of glomerulus in diabetic rats. Furthermore, diabetic glomerular damage in diabetic animal models responded to NO donor treatment by restoration of Wnt5a/ β -catenin signaling through downregulated oxidative stress and nitrosative stress pathways. We propose that restoration of NO levels might be of fundamental importance in the effect of NO donors to alleviate diabetic renal injury.

NO has been reported to modulate the vascular endothelial growth factor gene by accumulating hypoxia-inducible factor-1 α protein²⁵. Diabetes and hypoxia similarly activate the vascular endothelial growth factor gene transcription by the accumulation of hypoxia-inducible factor-1 α protein²⁶. However, diabetic renal fibrosis acts by a mechanism distinct from vascular endothelial growth factor-mediated diabetic proteinuria, and few previous studies have focused on the biological significance of NO donor treatment in diabetic fibrosis. In the present study, low urinary concentration of NO₂ and NO₃ was found to mediate high glucose-promoted apoptosis and extracellular matrix accumulation in 4- and 8-week animal models. These findings are based on the observation that the modulation of urinary NO levels can reduce apoptosis, and TGF- β 1 and fibronectin expressions of high glucose-stressed glomerulosclerosis. Furthermore, the optimal duration of NO donors for diabetic induction of renal injury was also one of our concerns. In our data, we have found that NO donors can more significantly alleviate diabetic kidney weight and proteinuria in 8 weeks of treatment when compared with those in 4 weeks of treatment. We cannot exclude the possibility that a NOC-18 concentration for 4 weeks could still elicit a protective effect for diabetic

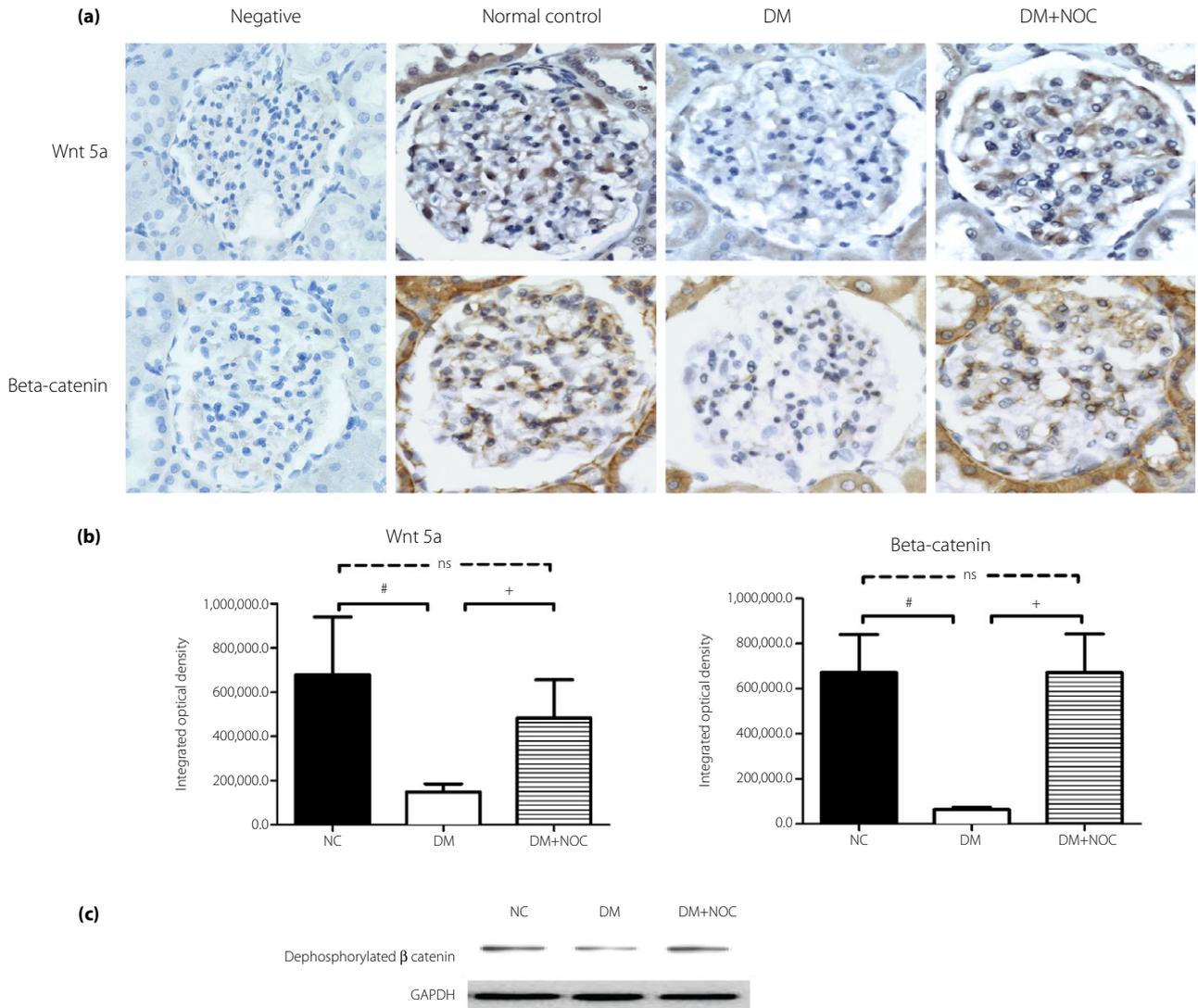


Figure 4 | (a) Immunohistochemical photograph of glomeruli in diabetic rats with and without nitric oxide (NO) donor (NOC) treatment. The diabetic group (DM) expressed weak Wnt5a and β -catenin expression when compared with the normal group (NC), and restored diabetes attenuation of Wnt5a and β -catenin expression by NO donor treatment. (b) Semi quantitative evaluation of the positive immune-labeled cells. (c) NO donor abrogated the suppressing effect of diabetes on dephosphorylated β -catenin expression. Data were expressed as mean \pm standard error. #*P* < 0.05 versus control group, +*P* < 0.05 vs NO donor treatment group. Specimens were observed under magnification $\times 400$.

nephropathy. However, the fact is that the protective effect on proteinuria occurs in 8 weeks, but not 4 weeks, despite the higher urinary NO₂ and NO₃ serum level in 4-week treatment. This shows that the positive actions of NO treatment are positively dependent on not only the amount of released NO, but also the duration of NO treatment in diabetic glomerular fibrosis and apoptosis.

Few previous studies have focused on the influence of NO donor treatment on high glucose-induced oxidative stress and nitrostatic stress on the kidney microenvironment *in vivo*. Redox control of TGF- β 1 and fibronectin expression in mesangial cells of the diabetic kidney have been reported in one of our previous studies¹⁵. Furthermore, the present study provides

immunohistochemical evidence that the glomerular cells in the diabetic kidneys showing the intensive 8-hydroxy-2'-deoxyguanosine associated with nitrostatic stress (ONOO⁻) immunostaining were reversed by NO donor treatment. Interestingly, NO donor treatment also completely restored eNOS activity in diabetic nephropathy. However, the NO donors restoring eNOS expression and/or activity in diabetic nephropathy have not yet been elucidated. We therefore suggest that 8 weeks of treatment with NO donors appears to upregulate the expression and the activity of the vascular eNOS pathway, resulting in not only a downregulation of oxidative enzymes, but also a direct scavenging of superoxide anion from the diabetic glomerular damage as a result of oxidative and nitrostatic stress. Previous study

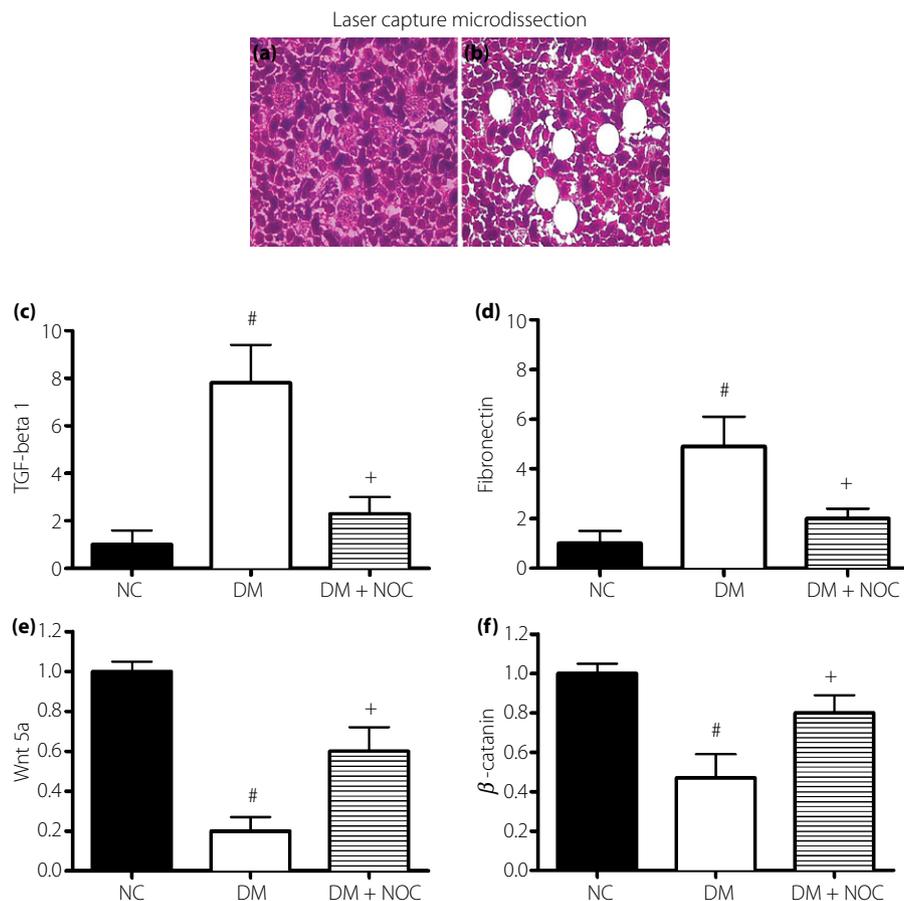


Figure 5 | Effect of nitric oxide (NO) donor (NOC) treatment on Wnt5a and fibrosis factor expression of the glomerular mesangium in diabetic kidneys. Representative histology photographs of renal tissue (a) before and (b) after dissection of glomerular mesangium. NO donor treatment attenuated (c) transforming growth factor (TGF)- β 1 and (d) fibronectin expression coincided with (e) reduced Wnt5a and (f) β -catenin messenger ribonucleic acid expression in the glomerular mesangium. Renal glomerular mesangium in rat kidneys were harvested by laser capture microdissection. DM, diabetic rats; NC, control group. Data were expressed as mean \pm standard error. [#] $P < 0.05$ vs control group, ⁺ $P < 0.05$ vs NO donor treatment group. Specimens were observed under magnification $\times 400$.

has shown that the genetic deletion of tumor necrosis factor receptor 1 in diabetic mice restored eNOS expression, which is associated with mitochondrial biogenesis. The NO donors diethylenetriamine nitric oxide and S-nitroso-N-acetyl-D,L-penicillamine prevented the reduction of mitochondrial biogenesis²⁷. As oxygen radical production is increased in diabetic glomerular damage, this NO donor-induced eNOS upregulation through the manipulation of mitochondrial biogenesis might play a key role in the renal protective effects on renal fibrosis and apoptosis. Furthermore, sustained NO donor treatment was associated with a decreasing peroxynitrite (ONOO⁻) in diabetic glomerulopathy, thus likely playing a major role in the development of alleviation of nitrostatic stress.

The present study, showing that NO donors rescue high glucose-induced downregulation Wnt/ β -catenin signaling in the glomeruli from diabetic rats, suggests an important mechanism for NO restoration in the regulation of Wnt signaling. We

previously showed that sustained Wnt signaling reduced c-Jun-dependent TGF- β 1-mediated fibronectin accumulation and caspase-3-dependent β -catenin-mediated apoptosis in diabetic glomerulopathy^{16,17,28}. These findings show that modulation of Wnt signaling, especially Wnt5a, is a foremost alternative strategy for rescuing the fibrotic and apoptotic signaling pathway in diabetic renal injury. We have previously shown that the Ras and Rac1 regulation of superoxide appeared to raise apoptotic activity by activating glycogen synthase kinase-3 β and inhibiting Wnt5a/ β -catenin signaling¹⁵. In the present study, we clearly showed that NO donor can significantly reduce superoxide-mediated oxidative stress. NO donor restoring Wnt/ β -catenin signaling through downregulation of superoxide in diabetic nephropathy could be the reason to elaborate this phenomenon. Thus, the increase in cellular β -catenin activity after NO donor treatment observed in the present study could indeed account for the effect on cellular fibrogenesis and

survival. Similarly, NO donor-treated diabetic rats also showed a reduction of albumin excretion, which is indicative of early diabetic renal injury. As such, the activity of the NO donor can be seen as intimately tied to the maintenance of Wnt/ β -catenin pathways in diabetic nephropathy. The findings of the present study can provide motivation for future investigations to study the effects of NO donors on Wnt/ β -catenin signaling. Furthermore, modulating Wnt/ β -catenin signaling transduction through NO donors might be an alternative strategy in the future for controlling diabetes by the reduction of Wnt/ β -catenin signaling, and renal cell apoptosis and fibrosis.

Taken together, the evidence of the present study suggests that NO donor treatment modulates the detrimental effects of high glucose in diabetic renal glomeruli *in vivo* by preventing the diabetes-mediated oxidative and nitrostatic stress, and restoring the downregulation of Wnt/ β -catenin signaling, thereby blocking the extracellular matrix accumulation and apoptosis in diabetic glomerulus. Furthermore, the present study also provides further evidence that the sustaining effect by NO donors on eNOS activity can rescue high glucose responsive TGF- β 1-stimulatory signal transduction in diabetic renal injury. These findings show that modulation of NO is a viable alternative strategy to rescue diabetic renal injury.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 | Biochemistry characteristics of rats with or without 2,2'-(hydroxynitrosohydrazino)bis-ethanamine treatment.