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Low nitric oxide bioavailability up-regulates renal heparin binding EGF-like growth factor expression

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Abstract

Decreased nitric oxide bioavailability plays an important role in the initiation and progression of diabetic nephropathy, but the underlying mechanisms remain unclear. Here, we found that heparin binding epidermal growth factor-like growth factor (HB-EGF) expression levels increased in the kidneys of both endothelial nitric oxide synthase (eNOS) knockout and eNOS knockout diabetic (Lepr db/db) mice as early as 8 weeks of age. Further increases in expression were only seen in eNOS knockout diabetic mice and paralleled the progression of glomerulopathy. HB-EGF expression increased in endothelium, podocytes, and tubular epithelial cells. In cultured glomerular endothelial cells, the nitric oxide synthase inhibitors NG-nitro-L-arginine methyl ester (L-NAME) or L-N5-(1-Iminoethyl) ornithine increased HB-EGF protein expression. Administration of L-NAME dramatically increased renal HB-EGF expression and urinary HB-EGF excretion in diabetic mice. On the other hand, replenishing nitric oxide with sodium nitrate in eNOS knockout diabetic mice reduced urinary HB-EGF excretion and inhibited the progression of diabetic nephropathy. Furthermore, specific deletion of HB-EGF expression in endothelium attenuated renal injury in diabetic eNOS knockout mice. Thus, our results suggest that decreased nitric oxide bioavailability leads to increased HB-EGF expression, which may be an important mediator of the resulting progressive diabetic nephropathy in eNOS knockout diabetic mice.

INTRODUCTION

Diabetes has become a pandemic disease, affecting both developing and developed countries. Diabetic nephropathy (DN), one of the major consequences of microangiopathy in

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DISCLOSURE

All the authors declared no competing interests.

diabetes, occurs in 20% to 40% of diabetic patients and is the leading cause of chronic kidney disease and end-stage renal disease (ESRD) in the United States. Therefore, measures to retard the progression of DN will have a significant impact on the overall morbidity, mortality and economic welfare of diabetic patients. Although clear progress has been made in understanding the disease process, there has been limited success in identifying specific factors that cause or predict nephropathy and its progression.

Endogenous NO is produced through the conversion of the amino acid, L-arginine to L-citrulline by NO synthases (NOS), of which there are three isoforms: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III). In endothelial cells, eNOS is the major source of NO, which plays an important role in vascular vasodilatation and the maintenance of vascular integrity.[1, 2] There is increasing evidence that decreased NO bioavailability in diabetes plays an important role in DN initiation and progression by causing endothelial dysfunction.

Previously, we have shown that eNOS deletion ($eNOS^{-/-}$) dramatically decreases glomerular NO production in both $eNOS^{-/-}$ only and $eNOS^{-/-}$ diabetic mice.[3] $Lepr^{db/db}$ mice with deletion of eNOS ($eNOS^{-/-}/Lepr^{db/db}$) have earlier and more severe renal function decline, as indicated by increased urinary albumin/creatinine ratio and decreased glomerular filtration rate (GFR), and exhibit more severe renal lesions such as mesangial expansion, focal nodular sclerosis and mesangiolysis compared to non-diabetic, $Lepr^{db/db}$, or $eNOS^{-/-}$ mice.[3,4] Accordingly, $eNOS^{-/-}/Lepr^{db/db}$ mice can be seen as a robust model for the study of type II diabetic nephropathy.

HB-EGF is a member of the EGF family of growth factors. Initially identified as a secreted product of cultured human macrophages,[5] it has subsequently been found to be expressed in other cell types including hematopoietic cells, endothelial cells, vascular smooth muscle cells and epithelial cells.[6–8] HB-EGF is mitogenic and chemotactic for those cells and has been implicated in a variety of physiologic and pathologic processes, including wound healing,[9] atherosclerosis,[10] blastocyte implantation,[11] tumor progression,[12] and glomerulonephritis.[13, 14] HB-EGF is synthesized as a transmembrane precursor protein (pro-HB-EGF) containing EGF-like and heparin-binding domains. Pro-HB-EGF can undergo proteolytic cleavage to release a mature soluble HB-EGF (sHB-EGF).[15, 16] We and others have demonstrated that both pro-HB-EGF and sHB-EGF are functionally active and can bind to and promote auto-phosphorylation of two members of the EGF receptor family, EGFR and ErbB4, by paracrine, autocrine and juxtacrine interactions.[17] Activated receptors then direct downstream signaling cascades with diverse biological effects, including cell proliferation, migration and differentiation.

HB-EGF stimulates eNOS expression and NO production in cultured endothelial cells.[18] Conversely, diabetic stimuli, including hyperglycemia, advanced glycation end products (AGEs) and oxidative stress, reduce NO production and increase HB-EGF expression in cultured cells and have been suggested to cause vascular damage not only by reacting with NO to form peroxynitrite or by uncoupling eNOS, but also through the direct actions of HB-EGF.[19–22] Thus, there may be a balance between NO levels and HB-EGF expression. In the

current studies, we examined the link between alterations in renal eNOS expression and HB-EGF expression in the eNOS$^{-/-}$/Lepr$^{db/db}$ model of diabetic nephropathy.

RESULTS

Increased HB-EGF expression in eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys

HB-EGF expression levels were higher in both eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys at 8 weeks of age, indicated by the 25 kDa band corresponding to pro-HB-EGF (Figure 1a and b), while minimal HB-EGF expression was detected in Lepr$^{db/db}$ or wild type (wt) mouse kidneys of the same age. Further increases in HB-EGF expression level were seen in eNOS$^{-/-}$/Lepr$^{db/db}$ mice at 16 weeks of age (Figure 1c and d) and later (data not shown). By 16 weeks of age, a 29 kDa higher molecular weight band was also detectable in some of the eNOS$^{-/-}$/Lepr$^{db/db}$ mice, consistent with expression of increased glycosylated forms of membrane-associated pro-HB-EGF.[23] Meanwhile, increased EGFR activation were detected in kidneys of diabetic, eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ mice compared to wild type mice (Figure 1e and f).

Higher urinary HB-EGF excretion in eNOS$^{-/-}$/Lepr$^{db/db}$ mice

Since pro-HB-EGF can be cleaved to release soluble HB-EGF, we determined whether renal HB-EGF was released into urine or serum. We failed to detect increased HB-EGF levels in the serum in either group (data not shown). However, higher amounts of HB-EGF were found in eNOS$^{-/-}$/Lepr$^{db/db}$ urine compared to other groups. HB-EGF excretion increased as early as 8 weeks of age (Figure 2a and c), and further increased at 16 weeks of age, with the 16 to 22 kDa molecular weight species corresponding to secreted HB-EGF (Figure 2b and d). In Lepr$^{db/db}$ or eNOS$^{-/-}$ non-diabetic mice, only trace or low levels of HB-EGF were detected in urine at 16 weeks of age (Figure 2b and d).

Increased HB-EGF expression was localized in glomeruli, endothelial cells, smooth muscle cells and tubular epithelial cells

Glomeruli from control mice had minimal staining for HB-EGF. Lepr$^{db/db}$, eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ all had detectable HB-EGF staining in the glomeruli, with the highest levels seen in eNOS$^{-/-}$/Lepr$^{db/db}$ mice. In arteries, HB-EGF staining was not detected in normal endothelial cells or smooth muscle cells (SMC). Although only weak staining was observed in the endothelial cells and SMC of Lepr$^{db/db}$ mice, strong HB-EGF staining was detected in endothelial cells of both eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ mice, and eNOS$^{-/-}$/Lepr$^{db/db}$ mice also had strong HB-EGF immunoreactivity in SMC (Figure 3a). In situ hybridization indicated strong HB-EGF mRNA expression in both glomeruli and tubular epithelial cells of eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys compared to other groups (Figure 3b).

In glomeruli of Lepr$^{db/db}$, eNOS$^{-/-}$, and eNOS$^{-/-}$/Lepr$^{db/db}$ mice, HB-EGF was detected primarily in podocytes, with lower expression levels in endothelial cells as indicated by double labeling of anti-HB-EGF with anti-podocin (a marker for podocyte) and anti-CD31 (a marker for endothelial cells) antibodies (Figure 3c and d).

In medulla, strong HB-EGF immunoreactivity was detected in tubules of both eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys, with less staining in Lepr$^{db/db}$ or wild type mouse kidneys (Figure 4a). Tubular-segment specific marker proteins indicated that HB-EGF localized to the apical membrane of the thick ascending limb and collecting duct epithelial cells without expression in proximal tubules (Figure 4b). Similar localization was observed by in situ hybridization (Figure 4c). There was minimal HB-EGF mRNA staining in the tubules of the wild type mouse kidneys.

NOS inhibition increased HB-EGF expression in glomerular endothelial cells (GENC)

The increased HB-EGF expression seen in eNOS$^{-/-}$/Lepr$^{db/db}$ mice kidneys suggested that decreased NO bioavailability may contribute to the increased HB-EGF levels. GENC were cultured for 48 hours in medium containing normal glucose (NG, 5.5 mM glucose), high glucose (HG, 30 mM glucose), mannitol (30 mM, used as a high osmolarity control for HG group), or the NOS inhibitors L-NAME (500 μM) or L-NIO (100 μM) with or without HG. Both L-NAME and L-NIO increased HB-EGF protein expression and EGFR phosphorylation after 48 hours, without any further augmented by administration of high glucose (Figure 5a, b, d and e). Since in vivo studies indicated that the highest HB-EGF expression was seen in eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys compared to either eNOS$^{-/-}$ or Lepr$^{db/db}$ mouse kidneys, these in vitro studies suggested that in eNOS$^{-/-}$/Lepr$^{db/db}$ mice, high glucose-related metabolic products rather than high glucose itself may contribute to the increased HB-EGF levels.

Up-regulation of HB-EGF expression by NO deficiency was also seen in another endothelial cell type. We detected much higher HB-EGF levels in cultured pulmonary endothelial cells (PEC) without eNOS expression compared to PEC with eNOS expression (cells provided by Mark de Caestecker, Vanderbilt University) (Figure 5c).

Development of albuminuria and up-regulation of HB-EGF expression in Lepr$^{db/db}$ mice after NO deprivation

L-NAME was administered to 12-week old Lepr$^{db/db}$ and wild type mice for 2 weeks. Systolic blood pressure (SBP) began to increase by 7 days of L-NAME injection (Figure 6a). There were no significant differences in blood pressure between Lepr$^{db/db}$ and wild type mice after L-NAME treatment. Albuminuria significantly increased in Lepr$^{db/db}$ mice treated with L-NAME, with increases after 1 week treatment, and further increases at 2 weeks treatment, while in the non-diabetic wild type mice, ACR remained unchanged after L-NAME treatment (Figure 6b). In Lepr$^{db/db}$ mice, L-NAME administration increased renal HB-EGF expression (Figure 6c and e). HB-EGF expression was not increased in the L-NAME treated non-diabetic wild type group in the observed period (data not shown). In addition, after L-NAME treatment, urinary HB-EGF excretion from the Lepr$^{db/db}$ mice was much higher compared to vehicle group, or either L-NAME or vehicle-treated wild type mice (Figure 6d and f).

NO replenishment with sodium nitrate (NaNO3) to eNOS$^{-/-}$/Lepr$^{db/db}$ mice slowed progression of renal injury and inhibited renal HB-EGF expression

In further studies, we determined the effects of NO replenishment in eNOS$^{-/-}$/Lepr$^{db/db}$ mice. For this purpose, NaNO3 was added to the drinking water, and renal pathology and HB-EGF expression were evaluated. Studies were begun at 8 weeks of age, at a time when eNOS$^{-/-}$/Lepr$^{db/db}$ mice already have increased albuminuria (24 h ACR: 487 ± 120 vs. 44 ± 3.3 in wild type mice). Although the degree of hyperglycemia was significantly lower in the NaNO3-treated group, there were no statistically significant differences in body weight or SBP (Table 1). The total NOx levels in both plasma and urine were significantly increased in NaNO3-treated mice versus control (Figure 7a and b), while the renal cGMP content did not show significant difference between NaNO3-treated mice and controls (2.67 ± 0.35 and 2.52 ± 0.33 pmol/mg protein, n = 8 in each group).

Compared to control, NaNO3 treatment markedly inhibited albuminuria. After 4 weeks of NaNO3 treatment, albuminuria was essentially stable for the subsequent 12 weeks (Figure 7c). After 16 weeks of NaNO3 administration, significant differences in renal morphology were observed. Compared to control mice of the same age, the NaNO3 treatment group had less glomerulosclerosis as shown by periodic acid Schiff (PAS) staining and assessed by sclerosis index (SI, 0 to 4 score, Figure 7d and e). The SI of the NaNO3 treatment group (1.40 ± 0.31) was significantly lower than the control group (2.32 ± 0.16, $P < 0.05$) (Figure 7e). Interstitial fibrosis was assessed by sirius red staining and found to be significantly reduced after NaNO3 treatment compared to control group (Figure 7d and f). Those results suggested that NO replenishment to the eNOS$^{-/-}$/Lepr$^{db/db}$ mice impeded overall renal deterioration.

To investigate the effect of NO replenishment on HB-EGF expression, we performed in situ hybridization. In 24-week old eNOS$^{-/-}$/Lepr$^{db/db}$ mice (control), HB-EGF mRNA was extensively expressed in the glomeruli and tubules, and this expression was significantly reduced after 16 weeks NaNO3 treatment (Figure 8a). Kidney HB-EGF protein expression levels were significantly less in the NaNO3 treatment group than in the control group (Figure 8b and d). Urinary HB-EGF excretion levels gradually decreased after NaNO3 treatment and were significantly lower after 12 weeks treatment (Figure 8c and e).

Specific HB-EGF deletion in endothelium attenuated diabetic nephropathy in streptozotocin (STZ)-induced diabetic eNOS$^{-/-}$ mice

Specific HB-EGF deletion in endothelium was confirmed by X-gal and HB-EGF staining at both 2 weeks and 20 weeks after tamoxifen injection (Figure 9a and b). In kidneys of 28-week old mice, glomerular sclerosis can be easily seen in diabetic eNOS$^{-/-}$ mice compared to eNOS$^{-/-}$/HB$^{endo-/-}$ mice (Figure 9c). Renal injury was significantly reduced in diabetic eNOS$^{-/-}$/HB$^{endo-/-}$ mice compared to diabetic eNOS$^{-/-}$/HB$^{lox/lox}$ mice as assessed by 24-hour urine albumin/creatinine ratio and glomerular injury score (Figure 9d and e).

DISCUSSION

The present study provides evidence that decreased NO bioavailability leads to up-regulation of HB-EGF expression in eNOS$^{-/-}$/Lepr$^{db/db}$ mice, a model of type II diabetic nephropathy. Increased renal HB-EGF expression was seen as early as 8 weeks of age in both eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys compared to wild type and Lepr$^{db/db}$ mice. HB-EGF immunoreactivity increased in glomeruli and endothelial cells of renal arteries. In cultured mouse glomerular endothelial cells, NOS inhibitors increased HB-EGF expression. Administration of the NOS inhibitor, L-NAME, increased renal HB-EGF expression in Lepr$^{db/db}$ mice with increased albuminuria. On the other hand, reduced HB-EGF expression by chronic NO replenishment to the eNOS$^{-/-}$/Lepr$^{db/db}$ BKS mice or specific HB-EGF deletion in endothelium of STZ-eNOS$^{-/-}$ C57BL/6 mice, which we have previously shown to accelerate diabetic nephropathy on this normally nephropathy resistant strain,[3] slowed diabetic nephropathy. Therefore, these results suggest increased renal HB-EGF expression in diabetes: a) results from alterations in NO bioavailability rather than hyperglycemia per se; and b) plays an important role in renal injury.

Although our studies indicate that initial increases in renal HB-EGF expression appear to be linked to reduced NO availability, the subsequent increases seen in eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys indicate that the diabetic milieu may also mediate further increases in HB-EGF expression. AGEs, increased oxidative stress[6,20], and increased angiotensin II levels have all been implicated in HB-EGF expression.[24] In this regard, there is evidence for increased oxidative stress and alterations in the renin-angiotensin system in kidneys of eNOS$^{-/-}$/Lepr$^{db/db}$ mice.[25]

In other kidney injury models such as puromycin aminonucleoside-induced nephrosis and ischemia-reperfusion (I/R) injury, increased HB-EGF expression was also detected in the distal nephron, including TAL and collecting ducts,[22,23] similar to the current localization seen in Lepr$^{db/db}$, eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$, with the highest expression levels in eNOS$^{-/-}$/Lepr$^{db/db}$ mice. Tubular casts with strong HB-EGF immunostaining were also observed in 16-week old eNOS$^{-/-}$/Lepr$^{db/db}$ mouse kidneys (data not shown). Although eNOS was first identified in vascular endothelium, it is widely expressed in many nephron segments, notably in TAL and collecting duct.[26]

Increased urinary HB-EGF excretion was seen predominantly in eNOS$^{-/-}$/Lepr$^{db/db}$ mice. HB-EGF localized to the apical surface of the tubular epithelial cells, even though basolateral localization could also be detected occasionally. In situ hybridization also showed HB-EGF mRNA expression in distal nephron, indicating that the source of the observed increases in tubular HB-EGF protein was more likely from local synthesis than from binding of secreted HB-EGF from the tubular fluid to membrane-associated proteoglycans. Progressive increases in urinary HB-EGF excretion in eNOS$^{-/-}$/Lepr$^{db/db}$ mice correlated with, and may be related to, underlying renal damage. Further studies will be required to determine whether the glomeruli or distal tubules are the source of the urinary HB-EGF.

Previous studies have implicated abnormalities in NO production in the development of diabetic nephropathy, although the underlying pathophysiologic mechanisms remain unclear. Although elevated NO levels were detected in both serum and urine after NaNO3 treatment, cGMP content in the kidney was not altered significantly. This is consistent with studies by Kumar et al. indicating that nitrite therapy did not induce significant increases in ischemic tissue cGMP levels. This group speculated that chronic nitrite therapy may increase ischemic tissue blood flow and stimulate arteriogenesis by modulating other endothelial cell signaling pathways such as Erk1/2 or PKC.[27]

In a recent study, Bollee et al. demonstrated a pathogenic role for HB-EGF activation of glomerular EGFR signaling in a mouse model of anti-GBM disease. Although elevated expression levels of EGFR were also observed in the glomeruli of eNOS$^{-/-}$/Lepr$^{db/db}$ mice (data not shown), the role of the HB-EGF–EGFR pathway in the development of DN in this model will require further investigation. However, the reverse correlation between HB-EGF expression and renal structural and functional alterations suggests that HB-EGF may be an important mediator of diabetic renal injury seen with decreased NO availability.

In conclusion, our results suggest that decreased NO bioavailability is a significant contributor to the increased HB-EGF expression in both eNOS$^{-/-}$ and eNOS$^{-/-}$/Lepr$^{db/db}$ mice, with further increases in HB-EGF expression secondary to the diabetic milieu. Under normal physiologic conditions, NO may negatively regulate HB-EGF expression, while with inhibition of NO expression and/or activity, loss of this negative regulation may contribute to increased HB-EGF expression, which might act as a mediator of glomerular damage.

CONCISE METHODS

Reagents and antibodies

D-(+)-glucose, D-mannitol, SIGMAFAST™ 3,3′-diaminobenzidine tablets, paraformaldehyde, streptozotocin, tamoxifen, rabbit anti-human podocin antibody, goat anti-mouse EGFR antibody, and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO); N^{G}-Nitro-L-arginine methyl Ester (L-NAME) and L-N5-(1-Iminoethyl) ornithine (L-NIO) were from EMD4Biosciences (Gibbstown, NJ). Heparin sepharose (HiTrap Heparin HP) was from GE Healthcare (Piscataway, NJ). Rabbit anti-rat HB-EGF polyclonal antibody was a gift from Lili Feng (Dept. of Immunology, The Scripps Research Institute, La Jolla, CA). Mouse anti-human HB-EGF and phospho-EGF receptor (Tyr1068) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

Other antibodies used in this study were rat anti-mouse CD31 (BD Pharmingen, Bedford, MA) and rabbit anti-human Tamm-Horsfall glycoprotein (THP) (Biomedical Technologies Inc. Stoughton, MA). Lotus tetragonolobus lectin (LTL), dolichos biflorus agglutinin (DBA), biotinylated goat anti-rabbit IgG, and avidin-biotin complex (ABC) kits were from VectorLabs (Burlingame, CA).

Animals

eNOS$^{-/-}$ mice on the C57BL/6J background, db heterozygous (Lepr$^{db/wt}$) mice on the C57BLKS/J (BKS) background and wild type BKS mice were purchased from The Jackson

Laboratory (Bar Harbor, ME). eNOS$^{-/-}$ mice were backcrossed for 10 generations to the BKS background and then crossed with Lepr$^{db/wt}$ mice to generate eNOS$^{-/-}$/Lepr$^{db/db}$ mice as previously described.[4] Genotypes were confirmed by PCR.

Mice with the HB-EGF gene flanked by loxP sites (HB$^{lox/lox}$) were kindly provided by Dr. Eisuke Mekada (Department of Cell Biology, Research Institute for Microbial diseases, Osaka University, Japan).[28] The targeting vector contained the lacZ gene as a reporter for the expression of HB-EGF. When HB-EGF cDNA is deleted by cre-recombinse, HB-EGF expression can be identified by X-gal staining in HB$^{+/-}$ or HB$^{-/-}$ mice. Homozygous HB$^{lox/lox}$ mice were bred with eNOS$^{-/-}$ to generate eNOS$^{-/-}$/HB$^{lox/lox}$ mice, which were further bred with mice that carry a tamoxifen-inducible Cre-ER(T) recombinase driven by the 5′ endothelial enhancer of the stem cell leukemia (SCL) locus (endothelial-SCL-Cre-ER(T), provided by Jimmy Hao, Vanderbilt University). 8-week old male littermates of eNOS$^{-/-}$/HB$^{lox/lox}$ and eNOS$^{-/-}$/HB$^{lox/lox}$/Cre(+) (eNOS$^{-/-}$/HB$^{endo-/-}$) mice were treated with STZ 50 mg/kg/day for five consecutive days to induce diabetes, and 2 mg tamoxifen was administered daily for five consecutive days to activate cre recombinase. Development of diabetes was evaluated by fasting blood glucose two weeks after the first injection, and those with blood glucose levels higher than 300 mg/dl were used for study. HB-EGF deletion was confirmed by X-gal staining and HB-EGF immunostaining. All the mice used in this part of the study were on C57BL/6 background. All animal experiments were performed under approval of the Institutional Animal Care and Use Committee at Vanderbilt University.

Blood pressure

SBP was measured in conscious trained mice at room temperature using a tail-cuff monitor (BP-2000 BP Analysis system, Visitech System, NC). Mice were subjected to 10 minutes per day of acclimation to the system under restrainers with tail cuffs for 4 days. On the fifth day, systolic blood pressure measurements were performed.

Urine albumin and creatinine

Urine from individual mice was collected in metabolic cages (Braintree Scientific, Braintree, MA) over 24 hours. Urine samples were centrifuged at 3,000 g for 5 minutes, and supernatants were stored in aliquots at −80°C until assay. Urinary albumin was determined using the Albuwell M Murine Microalbuminuria enzyme-linked immunosorbent assay kit (Exocell Inc., Philadelphia, PA). Urinary creatinine was measured using the Creatinine Companion kit (Exocell Inc.).

Urinary HB-EGF excretion

Excreted urinary HB-EGF was measured by a modification of previously described heparin affinity chromatography. Briefly, equal volumes of urine from 24-hour collections were added to heparin sepharose, incubated at 4°C overnight, washed 3 times with 10 mM phosphate buffer (pH 7.4), re-suspended, boiled in protein sample buffer before separation on 18% SDS-polyacrylamide gels, and followed by immunoblotting with an anti-HB-EGF antibody.

Cells and culture condition

GENC were provided by Dr. Michael Madaio, University of Pennsylvania, and condition. Cells were were cultured at 37°C with 5% CO_2 in a humidified airgrown to 80% confluence, starved for 24 hours in serum-free medium containing 0.1% bovine serum albumin (BSA), followed by incubation with mannitol (30 mM), D-Glucose (30 mM), L-NAME (500 μM), or L-NIO (100 μM) for 48 hours before harvesting for immunoblot analysis as described below.

In vivo L-NAME treatment

12-week old male $Lepr^{db/db}$ mice and non-diabetic BKS mice were given L-NAME (50 mg/kg) subcutaneously every day for two weeks. Sham groups were give equivalent amounts of normal saline. SBP was measured to monitor the effects of L-NAME.

In vivo NaNO3 treatment

NaNO3 was added to the drinking water of $eNOS^{-/-}/Lepr^{db/db}$ mice at a concentration of 85 mg/L (1 mM) beginning at 8 weeks of age for the treatment group and regular water for the control group. Urinary HB-EGF excretion levels and 24-hour urine albumin/creatinine ratios (ACR) were monitored at 4, 8, 12 and 16 weeks of treatment. Mice were sacrificed after 16 weeks of treatment and renal HB-EGF expression and pathological changes were examined. Plasma and urine nitrate/nitrite concentrations were determined using the Greiss reaction by measuring combined oxidation products of NO, nitrate (NO_3^-) and nitrite (NO_2^-), after reduction with nitrate reductase using a colorimetric assay (Cayman Inc., Ann Arbor, Michigan, USA). Determination of cGMP content was performed in acetylated samples using an immunoassay kit (Cayman Chemical Company, Ann Arbor, MI). cGMP levels were normalized to kidney protein content.

Renal pathology and immunostaining

Mice were euthanized at indicated ages and were perfused with ice cold normal saline, followed by 4% paraformaldehyde. Kidneys were removed and fixed overnight in 4% paraformaldehyde at 4°C, and 4 Lm thick sections were stained with PAS, sirius red or used for immunostaining. Alternatively, tissues were fixed in 4% paraformaldehyde for 2 hours at 4°C, washed with PBS, and put into 30% sucrose overnight at 4°C and then immersed into OCT for cryosectioning. Immunostaining was performed as previously described.[29]

Semiquantitative glomerulular injury and sclerosis index (0 to 4 score) was used to evaluate the degree of glomerulosclerosis, as previously prescribed.[30, 31] Tubular interstitial fibrosis was shown by sirius red staining and assessed by sirius red positive area. All sections were examined without knowledge of the treatment protocol.

Immunoblot analysis

A quarter of the kidney was snap-frozen and stored at −80°C until use. Frozen samples were homogenized and lysed in 0.5 ml ice-cold TGH buffer (1% Triton X-100, 10% glycerol, 20 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM Na_3VO_4). Western blots were conducted as

previously described. [29] Membranes were blotted using antibodies against HB-EGF, EGFR or phospho-EGFR.

In situ hybridization

Probes for HB-EGF were obtained by subcloning a 661-bp coding region of the mouse HB-EGF cDNA (NM 010415.2) into pCRII-TOPO vector (Invitrogen) and transcribing in vitro to produce antisense and sense riboprobes labeled with digoxigenin using DIG RNA labeling kit (Roche). In situ hybridization was performed as previously described.[32]

Statistical analysis

Data were evaluated using unpaired t-test for two-group comparison or one way ANOVA followed by Tukey-Krymer test for multiple comparisons and expressed as means ± SEM. Values of $P < 0.05$ were considered statistically significant. The effects of NaNO3 on metabolic and physiologic levels were examined using multivariable generalized least square regression analysis with adjustment for corresponding baseline levels (Table 1). Statistical analyses were performed using R version 2.15.1 (http://www.r-project.org). A two-sided significance level of 5% was required for consideration as statistical significant.

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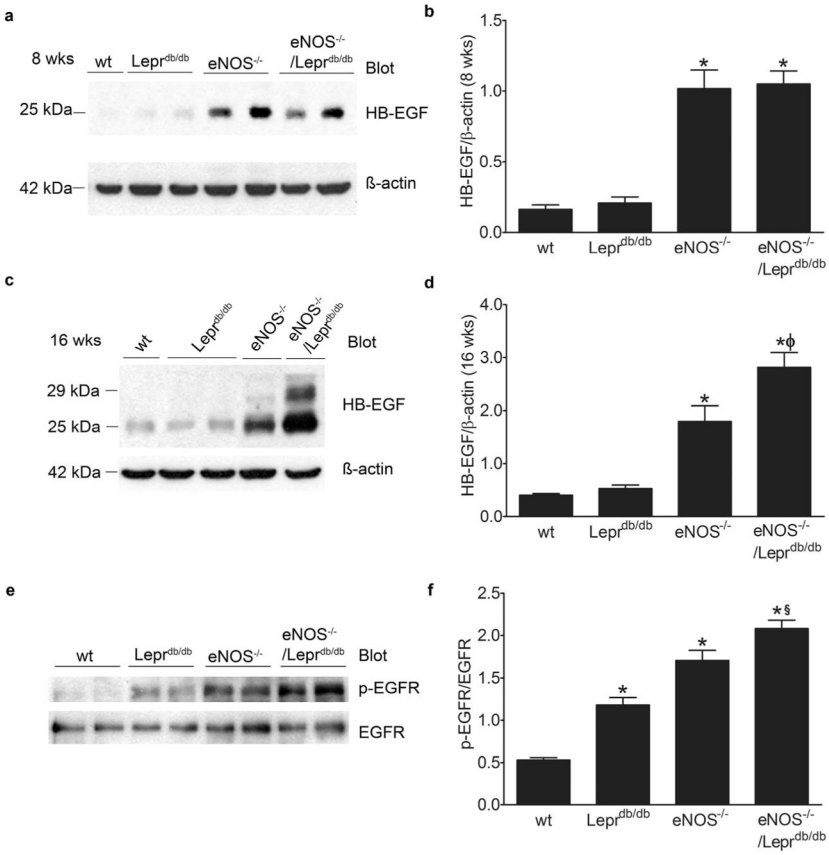

Figure 1. HB-EGF expression in mouse kidneys

Homogenates from kidney tissues of different group mice were separated by 12% SDS-PAGE and immunoblotted with anti-HB-EGF antibody. (**a**) Elevated HB-EGF expression level was seen in kidneys of 8-week old *eNOS*$^{-/-}$ and *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice. (**b**) Densitometric analysis of the Western blot bands in (**a**). HB-EGF expression level was normalized to its respective β-actin level and expressed as HB-EGF/β-actin. *$P < 0.05$ comparing to both wild type (wt) and *Lepr*$^{db/db}$ groups. (**c**) Increased HB-EGF level was present at age of 16 weeks in *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mouse kidneys with both 25 kDa and 29 kDa bands. (**d**) Densitometric analysis of the Western blot bands in (**c**) and expressed as HB-EGF/β-actin. *$P < 0.05$ comparing to both wt and *Lepr*$^{db/db}$ groups. *$P < 0.05$ comparing to eNOS$^{-/-}$ group. (**e**) Increased EGFR activation in the kidneys of diabetic, *eNOS*$^{-/-}$ and *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice compared to wild types. (**f**) Densitometric analysis of the Western blot bands in (**e**) and expressed as p-EGFR/EGFR. *$P < 0.05$ compared to wt. $^{§}P < 0.05$ compared to diabetic and eNOS$^{-/-}$ groups. Values represent the mean and SEM of three independent experiments (6 to 8 samples at each time point).

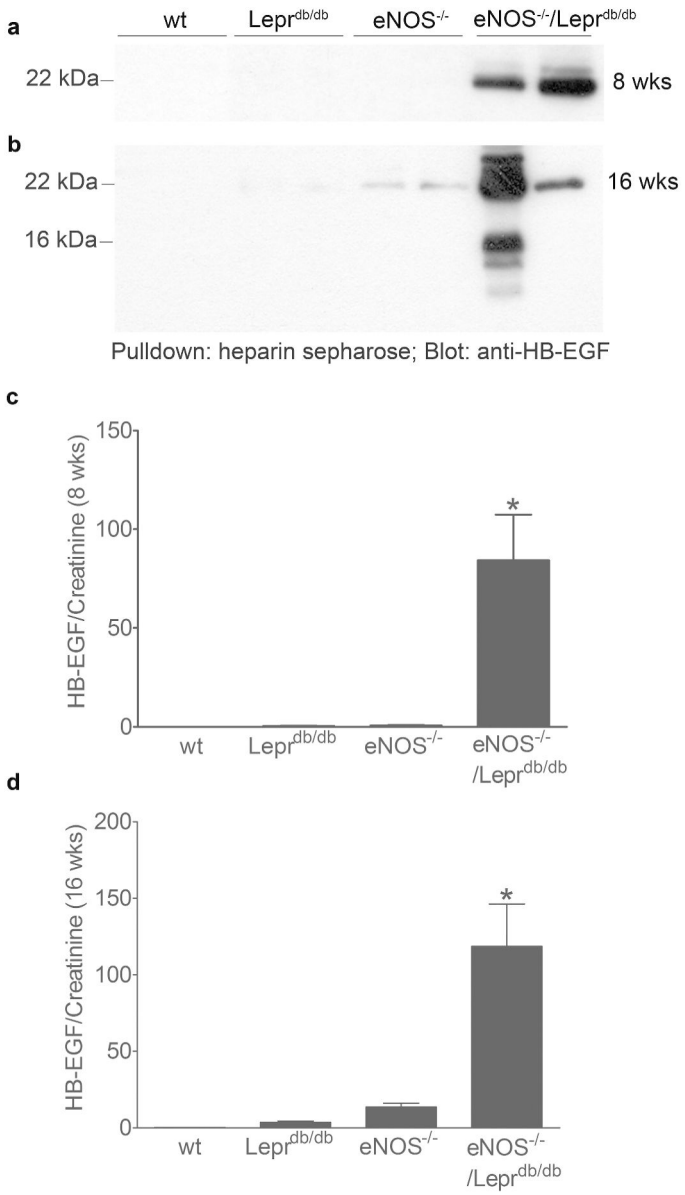

Figure 2. Urinary HB-EGF excretion levels

Urinary HB-EGF excretion levels were measured by a modified heparin-binding assay. Same amount of urine from 24-hour collection was added to the heparin sepharose and rotated overnight at 4ºC. HB-EGF was separated on an 18% SDS-PAGE and blotted using anti-HB-EGF antibody. (**a**) Significant amount of HB-EGF was detected in 8-week old *eNOS*$^{-/-}$*/Lepr*$^{db/db}$ mice only, with no detectable HB-EGF levels in other groups. (**b**) Increased urinary HB-EGF excretion at age 16 weeks were present in *Lepr*$^{db/db}$, *eNOS*$^{-/-}$, and *eNOS*$^{-/-}$*/Lepr*$^{db/db}$ mice, with the latter showing the highest level. Compared to 8 weeks, several additional bands of HB-EGF were detected, which represented different sizes of the secretion form of HB-EGF. (**c**) and (**d**) Densitometric analysis of the Western blot bands in (**a**) and (**b**). HB-EGF excretion level was normalized to its respective urinary

creatinine level and expressed as HB-EGF/creatinine. Mean values ± SEM are shown. *P < 0.05 comparing to three other groups.

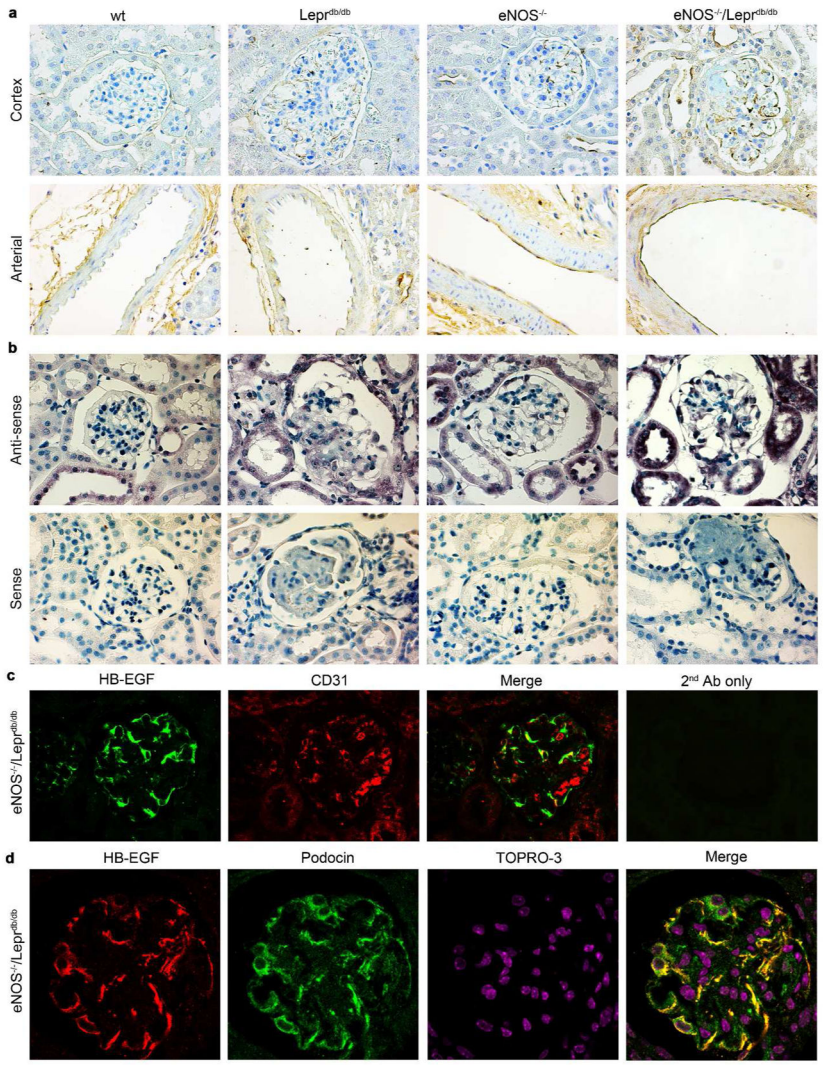

Figure 3. HB-EGF localization by immunohistochemistry

(**a**) Increased HB-EGF immunoreactivity was seen in *Lepr*$^{db/db}$, *eNOS*$^{-/-}$ and *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mouse kidneys, with *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ had the strongest immunostaining level. HB-EGF was localized in glomeruli, endothelial cells, and smooth muscle cells (brown color). X200. (**b**) *In situ* hybridization showed increased HB-EGF mRNA expression (purple) in both glomeruli and tubular epithelial cells in diabetic, *eNOS*$^{-/-}$ and *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice, with strongest expression in *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice. X630. (**c**) HB-EGF (green) was partially co-localized with CD31 (red), an endothelial cell marker, in the glomerulus of the *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice. X400. (**d**) HB-EGF expression in podocytes in *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice. HB-EGF (red) is co-localized with podocin (green), a podocyte marker, in the glomerulus of the *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice. TOPRO-3 (purple), nucleus staining. X800.

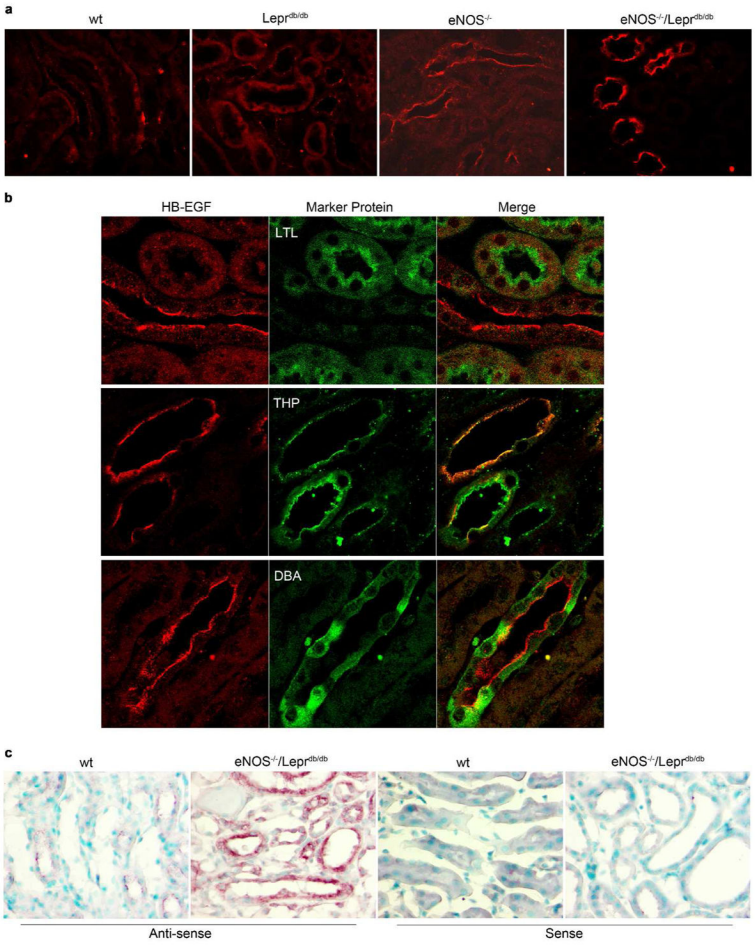

Figure 4. HB-EGF localization in tubules

(**a**) Increased HB-EGF immunoreactivity (red) was detected in the tubules of *eNOS*$^{-/-}$ and *eNOS*$^{-/-}$*/Lepr*$^{db/db}$ mice, compared to wt and *Lepr*$^{db/db}$ mice. X200. (**b**) Double immunofluorescence staining of HB-EGF with the indicated marker proteins demonstrated that HB-EGF is localized in the apical membrane of the thick ascending limb (co-localized with THP), distal tubule and collecting duct epithelial cells (co-localized with DBA) and is not expressed in proximal tubules (no co-localization with LTL). HB-EGF: red; marker proteins: green. X400. (**c**) HB-EGF mRNA level by *in situ* hybridization. Wild type mice showed low level HB-EGF mRNA expression in the tubules, with increased HB-EGF signal in *eNOS*$^{-/-}$*/Lepr*$^{db/db}$ mouse kidneys. Red, HB-EGF; green, methylene green for nucleus. X200.

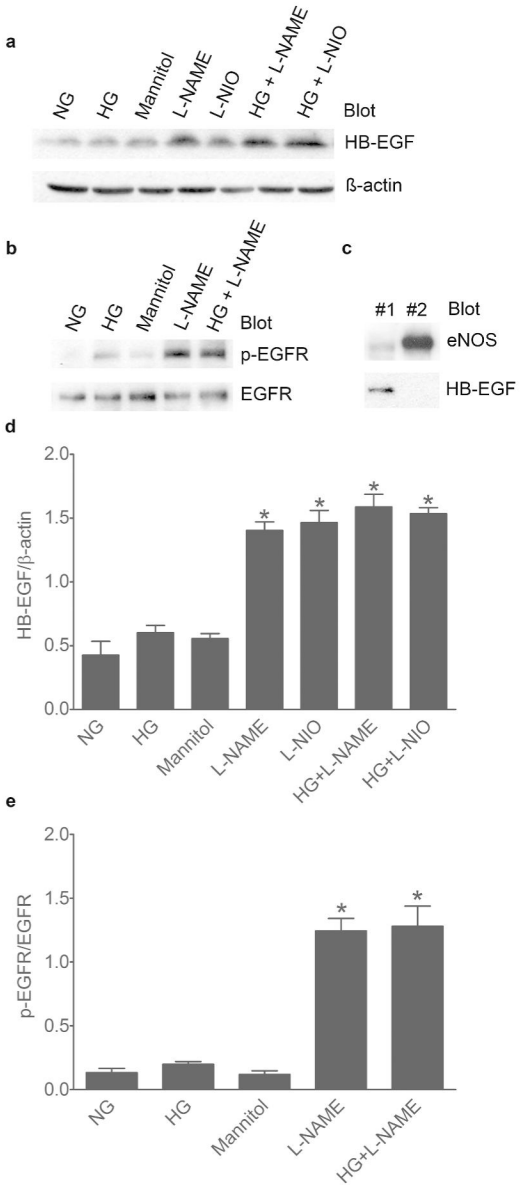

Figure 5. NOS inhibition up-regulated HB-EGF expression and EGFR activation in endothelial cells

(**a**) HB-EGF expression levels in glomerular endothelial cells (GENC) by Western blot. High concentration glucose (HG) and mannitol did not alter HB-EGF expression levels in GENC. Both NOS inhibitors, L-NAME or L-NIO, increased HB-EGF expression after 48 hours treatment, with no further elevation by additional HG. (**b**) Increased EGFR phosphorylation in L-NAME and HG plus L-NAME treatment cells compared to all other groups. (**c**) Increased HB-EGF expression was seen in pulmonary endothelial cells (PEC) with minimal detectable eNOS level (#1), while no HB-EGF was detected in PEC with high level of eNOS expression (#2). (**d**) Densitometric analysis of the Western blot bands in (**a**). HB-EGF expression level was normalized to its respective β-actin level and expressed as HB-EGF/β-actin. **P* < 0.05 comparing to normal glucose control group. (**e**) Densitometric

analysis of the Western blot bands in (b). Phospho-EGFR level was normalized to EGFR level and expressed as p-EGFR/EGFR. *$P < 0.05$ compared to groups without NOS inhibitor treatment. Values represent the mean and SEM of three independent experiments.

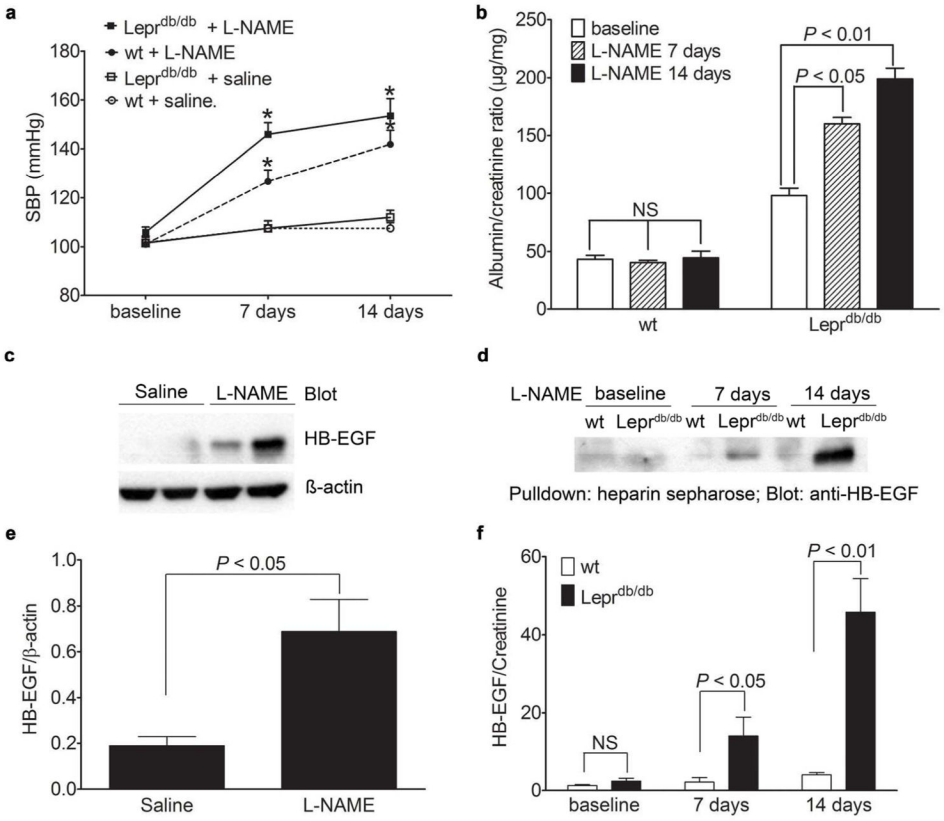

Figure 6. Effects of L-NAME on renal function and HB-EGF expression *in vivo.*< br>**(a)**L-NAME administration rapidly increased systolic blood pressure (SBP) in wild type (wt) and *Lepr*$^{db/db}$ groups in a short period of time, and reached significant difference compared to vehicle groups (*$P < 0.05$, means ± SEM of 8 samples in each group). **(b)** In *Lepr*$^{db/db}$ mice, urinary albumin/creatinine ratio (ACR) increased significantly after 7 days L-NAME administration, and increased further by 14 days. ACR did not change in wild type mice after L-NAME treatment. **(c)** Compared to vehicle group, HB-EGF expression levels were increased significantly after 14 days L-NAME administration in *Lepr*$^{db/db}$ mouse kidneys. **(d)** Significant increase of urinary HB-EGF excretion was seen only in *Lepr*$^{db/db}$ mice after 14 days of L-NAME administration. **(e)** Densitometric analysis of the Western blot bands in (c). HB-EGF expression level was normalized to its respective β-actin level and expressed as HB-EGF/β-actin. Mean values ± SEM are shown. **(f)** Densitometric analysis of the Western blot bands in (d). HB-EGF excretion level was normalized to its respective urinary creatinine level and expressed as HB-EGF/creatinine. Mean values ± SEM are shown.

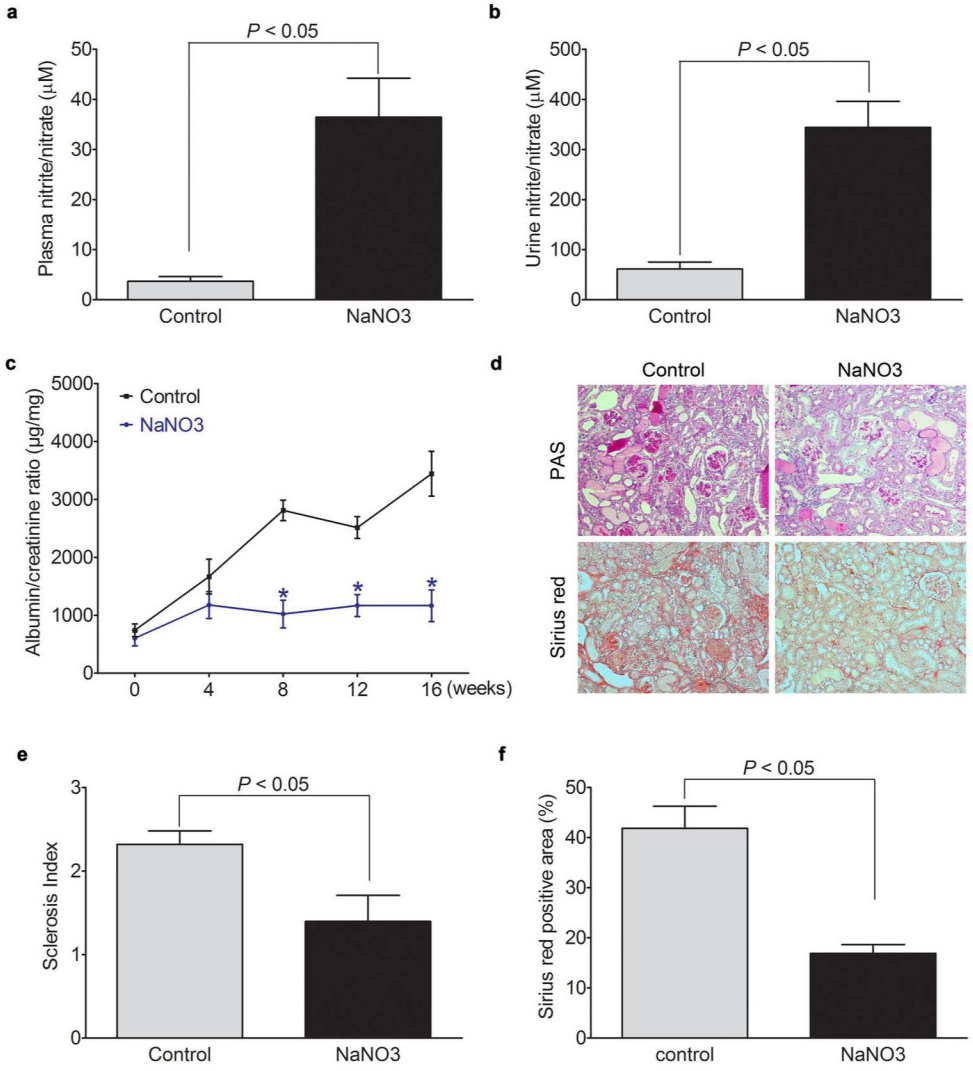

Figure 7. Effects of NaNO3 administration on *eNOS*$^{-/-}$/*Lepr*$^{db/db}$ mice

(**a–b**) After 4 weeks of NaNO3 administration, both plasma and urinary nitrite/nitrate concentration (μM) were significantly increased in NaNO3 treatment mice comparing to control group. (**c**) 24-hour urine albumin/creatinine ratio (ACR) changes. Compared to control, NaNO3 administration stabilized ACR level after 4 weeks, and reached significant difference 8 weeks later (*$P < 0.05$, mean ± SEM of 8 to10 samples in each group). (**d**) Renal pathological changes. Compared to control, NaNO3 treated mice at age 24 weeks has milder degree of glomerular sclerosis as shown by PAS staining and less degree of interstitial fibrosis shown by sirius red staining. X200. (**e**) Glomerular sclerosis shown by PAS staining in (**d**) was assessed by sclerosis index score (0–4) and shown as mean values ± SEM of 8 to 10 samples in each group. (**f**) Interstitial fibrosis shown by sirius red staining in (**d**) was assessed by sirius red positive area and shown as mean values ± SEM of 8 to 10 samples in each group.

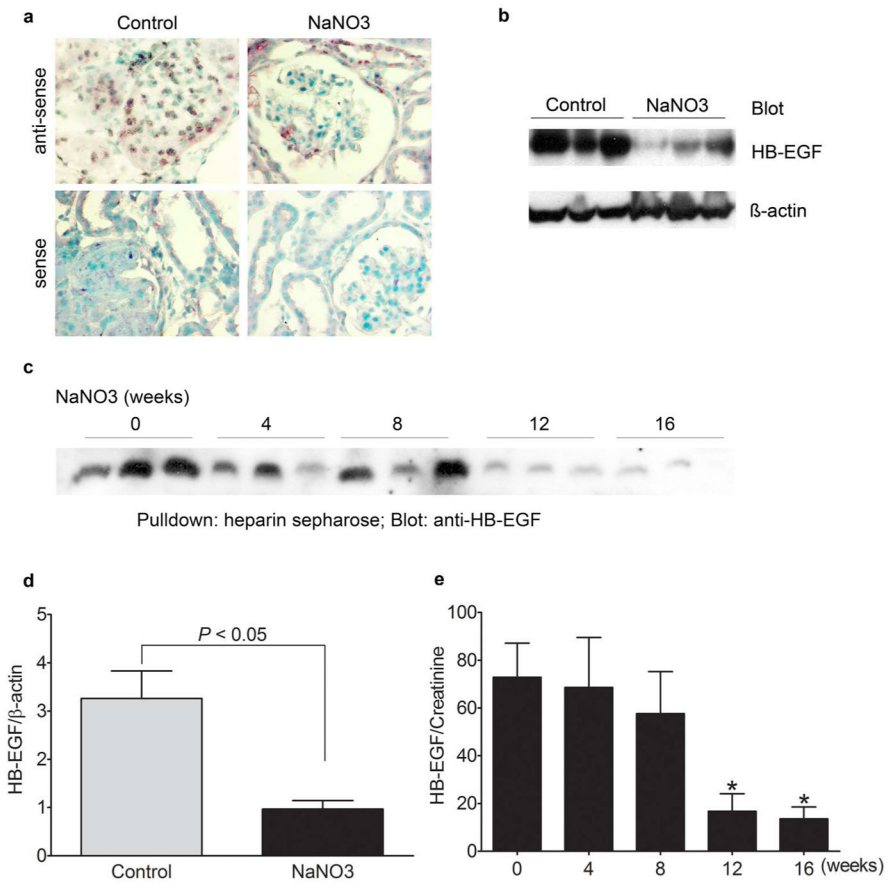

Figure 8. Effect of NaNO3 on HB-EGF expression level

(**a**) HB-EGF mRNA level by *in situ* hybridization. NaNO3 treatment decreased HB-EGF mRNA expression level, especially in glomeruli. Red, HB-EGF; green, methylene green. X400. (**b**) Kidney HB-EGF protein expression levels by Western blot were significantly decreased after NaNO3 administration in $eNOS^{-/-}/Lepr^{db/db}$ mice. (**c**) Urinary HB-EGF excretion remained stable in the first 8 weeks of NaNO3 treatment, followed by decreased HB-EGF excretion levels after 12 weeks treatment. (**d**) Densitometric analysis of the Western blot bands in (**b**). HB-EGF expression level was normalized to its respective β-actin level and expressed as HB-EGF/β-actin. (**e**) Densitometric analysis of the Western blot bands in (**c**). Urinary HB-EGF excretion level was normalized to its respective urinary creatinine level and expressed as HB-EGF/creatinine. Mean values ± SEM of 6 to 8 samples on each time point are shown. *$P < 0.05$ comparing to 0, 4 and 8 weeks of NaNO3 treatment.

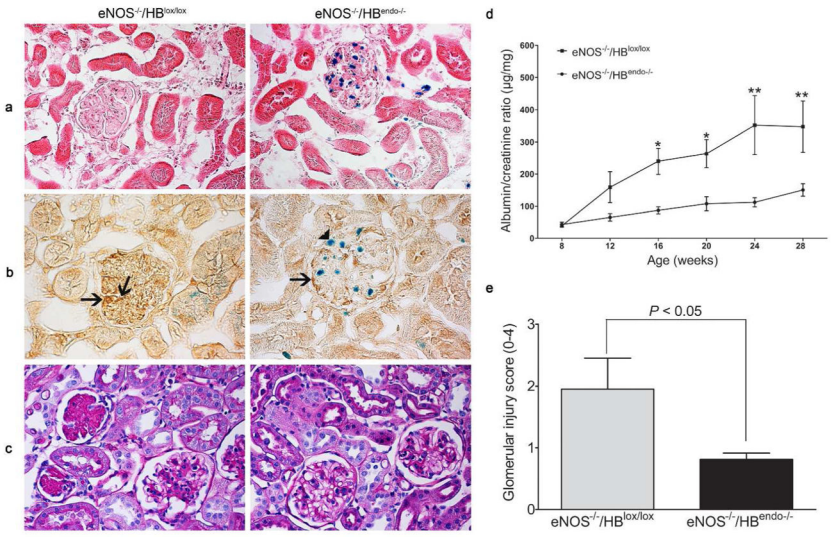

Figure 9. The effect of selective endothelial deletion of HB-EGF on renal histopathological and functional changes in diabetic eNOS$^{-/-}$ mice

(**a**) X-gal staining (blue) showed that HB-EGF was expressed and being deleted in glomerular and peritubular endothelium of 28-week old diabetic eNOS$^{-/-}$/HB$^{endo\ -/-}$ mouse kidneys. Counterstain: sirus red. X400. (**b**) Double staining for HB-EGF (brown, arrow) and X-gal (blue, arrow head) showed that HB-EGF was deleted from glomerular endothelium in diabetic eNOS$^{-/-}$/HB$^{endo\ -/-}$ mice compared to diabetic eNOS$^{-/-}$/HB$^{lox/lox}$ mice. X600. (**c,e**) Glomerular sclerosis and/or masangial expansion was determined by assessing the glomerular injury score of PAS staining samples. X400. (**d**) Reduced albumin/creatinine ratio in diabetic eNOS$^{-/-}$/HB$^{endo\ -/-}$ mice compared to diabetic eNOS$^{-/-}$/HB$^{lox/lox}$ mice. n = 8 in each group. * $P < 0.05$; ** $P < 0.01$.

Table 1

Metabolic and physiologic parameters

NANO3 (W EEKS)	0 (BASELINE)	4	8	12	16	*P*
BW (g)						
Control	30.6±1.6	41.3±1.1	48.5±1.4	47.8±2.4	51.4±3.9	0.68
NaNO3	28.6±0.8	37.8±1.9	45.7±2.6	48.0±3.0	50.6±5.3	
BG (mg/dl)						
Control	263.8±38.1	390.8±33.9	490.7±34.1	519.7±30.4	477.5±33.1	0.003
NaNO3	276.3±48.1	393.9±41.2	403.6±39.5	383.9±70.0	411.2±77.0	
SBP (mmHg)						
Control	126.0±6.0	131.8±3.3	148.3±5.7	167.0±6.6	157.2±7.4	0.62
NaNO3	123.4±3.7	126.8±7.4	136.3±4.5	146.8±4.0	155.3±4.5	

Values are shown as means ± SEM of 8 to 10 samples in each measuring point. BW, body weight; BG, blood glucose; SBP, systolic blood pressure. P value is from multivariable generalized least square regression analysis assessing the effect of NaNO3 treatment on metabolic and physiologic levels with adjustment for corresponding baseline measurements.