Endothelial dysfunction promotes the transition from compensatory renal hypertrophy to kidney injury after unilateral nephrectomy in mice

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Endothelial dysfunction promotes the transition from compensatory renal hypertrophy to kidney injury after unilateral nephrectomy in mice. Am J Physiol Renal Physiol 302: F1402–F1408, 2012. First published February 27, 2012; doi:10.1152/ajprenal.00459.2011.—Loss of functional nephrons associated with chronic kidney disease induces glomerular hyperfiltration and compensatory renal hypertrophy. We hypothesized that the endothelial nitric oxide synthase (eNOS) [soluble guanylate cyclase (sGC)] protein kinase G (PKG) pathway plays an important role in compensatory renal hypertrophy after unilateral nephrectomy. Analysis of mice subjected to unilateral nephrectomy showed increases in kidney weight-to-body weight and total protein-to-DNA ratios in wild-type but not eNOS knockout (eNOSKO) mice. Serum creatinine and blood urea nitrogen increased after nephrectomy in eNOSKO but not in wild-type mice. Furthermore, Bay 41–2272, an sGC stimulator, induced compensatory renal hypertrophy in eNOSKO mice and rescued renal function. The NO donor S-nitrosoglutathione (GSNO) and Bay 41–2272 stimulated PKG activity and induced phosphorylation of Akt protein in human proximal tubular cells. GSNO also induced phosphorylation of eukaryotic initiation factor 4E-binding protein and Akt protein in human proximal tubular cells. GSNO also induced phosphorylation of eukaryotic initiation factor 4E-binding protein and ribosomal protein S6. Our results highlight the importance of the eNOS-NO-PKG pathway in compensatory renal hypertrophy and suggest that reduced eNOS-NO bioavailability due to endothelial dysfunction is the underlying mechanism of failure of compensatory hypertrophy and acceleration of progressive renal dysfunction.

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Mice were killed after a 2-wk observation period. The kidney was weighed and corrected for body weight. Blood samples were obtained using a 23-gauge needle inserted into the right ventricle at the time of death for measurement of serum creatinine (CRN) and blood urea nitrogen (BUN) levels using enzymatic methods (9). The left ventricle was cannulated, and the kidney was perfused with ice-cold PBS (pH 7.4). Half of the removed kidneys were immersed and fixed in 4% paraformaldehyde and then embedded in paraffin for periodic acid-Schiff staining. The 24-h urine samples were collected the day before death. Urinary albumin (U-ALB) was quantified by Albuwell M (Exocell, Philadelphia, PA), and the albumin-to-creatinine ratio was calculated. Urinary nitrite/nitrate (NOx) excretion was determined by a colorimetric method with the Griess reaction, using a BIOMOL Nitric Oxide Assay Kit (BIOMOL Research Laboratories, Plymouth Meeting, PA) (14).

Histopathological examination. Paraffin-embedded kidney sections (~4 μm thick) were deparaffinized and stained with periodic acid-Schiff staining.

Measurement of protein/DNA ratios in vivo. The renal cortex was homogenized in 1 ml lysis buffer (0.02% SDS, 150 mM NaCl, and 15 mM Na citrate) followed by a 10-fold dilution. DNA concentrations were measured in triplicate as described previously (19). Briefly, aliquots of each homogenate were incubated in a 96-well plate at 37°C for 1 h. After the addition of 100 μl 1.0 μg/ml bisbenzimidazole fluorescent dye Hoechst 33258 (Sigma-Aldrich Japan), the samples were read at excitation λ360 nm and emission λ460 nm using a CytoFluor II spectrophuorometer (PerSeptive Biosystems, Cambridge, MA). Aliquots of the same homogenates were used to determine total protein concentration by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). The protein-to-DNA ratio was calculated, and data were presented as percentage increases relative to WT sham.

Human proximal tubular cells. Normal human proximal tubular cells (hPTECs; Cell Systems, Kirkland, WA) were maintained in renal epithelial basal medium containing REGM SingleQuots, 5% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO2. Confluent cells passaged 7–10 times were serum starved overnight in 1% FBS, stimulated with 500 μM of the NO donor S-nitrosothiolamine (GSNO; Sigma-Aldrich) (28) and 10 μM Bay 41–2272 (30), and incubated for 30 min. Cells were then harvested to determine PKG activity and the mTOR pathway.

Measurement of protein-to-cell counts ratios in vitro. hPTECs were homogenized in ice-cold 1% FBS buffer containing protease inhibitors and centrifuged at 8,000 g, and the supernatants were assayed for mTOR activity by Western blotting analysis. Cells were homogenized on ice in 1 ml lysis buffer containing protease inhibitors and centrifuged at 8,000 g for 10 min. The protein concentration of the supernatants was determined with a Bio-Rad protein assay kit (CycLex, Naganawa, Japan) (17). Samples were boiled in appropriate extraction buffer (in mM: 20 Tris, pH 7.4, 150 NaCl, 1 EDTA, 1 EGTA, 0.2 PMSF, 2 NaF, 0.2 Na3VO4, 5 β-mercaptoethanol, and 1 μg/ml pepstatin, 0.5 μg/ml leupeptin), and resuspended cells were lysed using three cycles of freezing and thawing. The upper lysate was measured using a peroxidase-coupled anti-phospho-G-kinase threonine 68/119 monoclonal antibody as a reporter molecule.

RNA isolation and real-time quantitative PCR. Total RNA was isolated from the glomeruli with Trizol (Invitrogen Japan, Tokyo, Japan). Reverse transcriptase reactions were performed using a Ready-To-Go T-Primed First-Strand kit (GE Healthcare Bio-Sciences, Tokyo, Japan) for first-strand cDNA synthesis. Real-time quantitative PCR was performed using the ABI Prism 7700 sequence-detection system (Applied Biosystems, Foster City, CA). Primers and probes for TαqMan analysis were designed using Primer Express 1.5 (Applied Biosystems) with information from the supplier based on the sequence information from GenBank or EST databases. The primers and probes for eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS) were as follows: eNOS, 5'-CAGGCATCACCCAGGAGAA-GAA-3' (forward primer); 5'-GAATGTTGCTCCCTCAGCG-3' (reverse primer); and 5'-FAM-ATCTCTGCTCCTCATGCGACCG-TAMRA-3' (TaqMan probe). iNOS, 5'-AGacctacagagactcct-3' (forward primer); 5'-ggctcctccctgc-3' (reverse primer); and 5'-FAM-cattgagctgaaattcgc-a-TAMRA-3' (TaqMan probe). nNOS, 5'-ggcgaactcctctcaca-3' (forward primer); 5'-tggagactctcgc-3' (reverse primer); and 5'-FAM-cattgagctgaaattcgc-a-TAMRA-3' (TaqMan probe).

Measurement of mTOR activity by Western blotting analysis. Cells were homogenized on ice in 1 ml lysis buffer containing protease inhibitors and centrifuged at 8,000 g for 10 min. The protein concentration of the supernatants was determined with a Bio-Rad protein assay kit, and 40 μg of protein was subjected to Western blot analysis with appropriate antibodies to detect the levels of phosphorylated Akt, total Akt, phosphorylated eukaryotic initiation factor 4E-binding protein (4EBP), total 4EBP, phosphorylated ribosomal protein S6 (rpS6) and total rpS6 (all antibodies from Cell Signaling Technology, Beverly, MA). Signals were visualized with ECL Western Blotting Detection Reagents (Amersham), captured on Hyperfilm ECL (Amersham), and scanned and quantified by densitometry (GE Healthcare Bio-Sciences, Tokyo, Japan). The integrated density (density 3 area) of the bands was quantified using Image-J software (http://rsbweb.nih.gov/ij/) (20).

### Table 1. Physiological and biochemical data at 2 wk after nephrectomy

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>NOSKO</th>
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<th>WT</th>
<th>NOSKO</th>
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<th></th>
<th>NOSKO</th>
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<tr>
<td>BP, mmHg</td>
<td>98 ± 6</td>
<td>96 ± 5</td>
<td></td>
<td>118 ± 4</td>
<td>116 ± 6</td>
<td></td>
<td>110 ± 6</td>
<td>112 ± 8</td>
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<tr>
<td>BUN, mg/dl</td>
<td>25 ± 3</td>
<td>31 ± 3</td>
<td></td>
<td>26 ± 2</td>
<td>56 ± 5*†</td>
<td></td>
<td>27 ± 2</td>
<td>34 ± 5</td>
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<tr>
<td>CRN, mg/dl</td>
<td>0.08 ± 0.01</td>
<td>0.11 ± 0.03</td>
<td></td>
<td>0.10 ± 0.02</td>
<td>0.14 ± 0.02*†</td>
<td></td>
<td>0.08 ± 0.03</td>
<td>0.09 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>U-ALB, mg/g:CRN</td>
<td>186 ± 10</td>
<td>192 ± 12</td>
<td></td>
<td>170 ± 16</td>
<td>240 ± 13*†</td>
<td></td>
<td>174 ± 22</td>
<td>196 ± 18</td>
<td></td>
</tr>
<tr>
<td>U-NOx, mg/g:CRN</td>
<td>10 ± 0.5</td>
<td>13.5 ± 0.8*</td>
<td></td>
<td>7.2 ± 0.2</td>
<td>8.2 ± 0.3*</td>
<td></td>
<td>7.0 ± 0.2</td>
<td>8.6 ± 0.4*</td>
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</table>

Data are means ± SD. WT, wild-type mice; eNOSKO, eNOS knockout mice; Nx, nephrectomy; Bay, Bay 41–2272; BP, blood pressure; BUN, blood urea nitrogen; CRN, serum creatinine; U-ALB, urinary albumin excretion; U-NOx, urinary nitrite/nitrate excretion. *P < 0.05 vs. correspondent sham. †P < 0.05 vs. WT Nx.
Statistical analysis. Values are expressed as means ± SE. Differences between groups were examined for statistical significance using a two-tailed unpaired Student’s t-test or one-way ANOVA for comparison of multiple means. A P value < 0.05 was considered statistically significant.

RESULTS

Hemodynamic response after nephrectomy in eNOS-deficient mice. Renal blood flow was increased 1.3 times compared with pregilation in WT mice (Fig. 1). However, there was no significant change between pre- and postligation values in eNOSKO mice.

Compensatory renal hypertrophy in eNOS-deficient mice. The significance of the eNOS-sGC pathway in compensatory renal hypertrophy was investigated in WT and eNOSKO mice. Table 1 lists the physiological and biochemical data measured at 2 wk after Nx or sham operation. Serum CRN and BUN increased significantly after Nx in eNOSKO but not WT mice. U-ALB did not increase after Nx in WT mice, while there were significant differences between eNOSKO-sham and eNOSKO-Nx mice. U-ALB levels were partially suppressed in eNOSKO-Nx mice following the administration of Bay 41–2272. Urinary NOx levels increased significantly after Nx in WT mice, but did not change after the administration of Bay 41–2272 in eNOSKO mice.

The size of the glomeruli was smaller in eNOSKO than WT mice, but there were no pathological changes in the interstitium or glomerular region, such as inflammatory cell infiltration or fibrotic changes (data not shown). We used the kidney weight-to-body weight and total protein (TP)/DNA ratios as markers of cellular hypertrophy in the kidney. Both ratios increased after Nx in WT mice (Fig. 2, A and B) but not in eNOSKO mice, suggesting that compensatory renal hypertrophy is mediated via eNOS-derived NO. Bay 41–2272 significantly increased the kidney weight-to-body weight ratio after Nx in eNOSKO mice. Furthermore, the TP/DNA ratio was also significantly higher in eNOSKO-Nx +Bay compared with eNOSKO sham +Bay 41–2272 mice. These effects of Bay 41–2272 in eNOSKO-Nx were completely suppressed by co-administration of rapamycin. This indicates that activation of sGC-mTOR even without eNOS can induce compensatory renal hypertrophy.

Enhanced compensatory hypertrophy in EC-eNOSTG. We also used EC-eNOSTG mice to investigate the role of eNOS in compensatory renal hypertrophy. The kidney weight-to-body weight and TP/DNA ratios were significantly higher in EC-eNOSTG-sham compared with WT-sham mice (Fig. 2C). Furthermore, the kidney weight-to-body weight ratio was significantly higher in EC-eNOSTG-Nx mice than WT-Nx mice (Fig. 2C). The TP/DNA ratio also increased after Nx in EC-eNOSTG mice compared with WT mice. These findings suggest that enhanced activation of eNOS boosts compensatory hypertrophy.

Several NOS isoforms assessed by real-time PCR in kidney. Figure 3 shows several isoforms of NOS expression in kidney. Expression of eNOS was increased after Nx in WT and EC-eNOSTG mice (Fig. 3A). Expression of nNOS mRNA was increased in eNOSKO compared with WT (Fig. 3B). But this expression was decreased after Nx in WT, eNOSKO, and EC-eNOSTG mice. iNOS expression was not significantly changed after Nx in all groups (Fig. 3C).

NO donor-stimulated Akt-mTOR pathway. To determine whether NO stimulates protein synthesis in proximal tubules, we investigated activation of the Akt-mTOR pathway. hPTECs were used as a standard. *P < 0.05. eNOSKO, eNOS-deficient mice. Bay 41–2272 was used as a sGC stimulator. C: KW/BW and TP/DNA ratios. Data are expressed relative to the values of WT-sham animals, which were used as a standard. Data are means ± SD of 10 animals. *P < 0.05. EC-eNOSTG, endothelial-specific eNOS transgenic mice.
UNx impaired renal function and induced albuminuria as a result of maladaptation. Bay 41–2272, a sGC stimulator, restored renal function and reduced urinary albumin excretion through the activation of sGC in eNOS-deficient mice, whereas all of these effects were suppressed following administration of the mTOR inhibitor, rapamycin. These findings indicate that impairment of NO availability associated with endothelial dysfunction promotes the switching from compensatory renal hypertrophy to kidney damage.

NO is a potent vasodilator produced by endothelial cells and known to mediate vascular relaxation induced by vasodilators (e.g., acetylcholine, bradykinin and substance P), and lowering tubular cell hypertrophy. Similar to GSNO, Bay 41–2272 stimulated PKG activity in hPTECs compared with untreated cells (Fig. 5A). Figure 5B shows representative Western blots for phospho-Akt and total Akt with or without Bay 41–2272 treatment. Bay 41–2272 induced phosphorylation of Akt protein in hPTECs. Similar to GSNO, Bay 41–2272 induced phosphorylation of 4EBP and rps6 (Fig. 5, C and D). Figure 5E shows assessed cell hypertrophy by using total protein-to-cells ratio. Bay 41–2272 induced cell hypertrophy in vitro, and these changes were suppressed by rapamycin.

**DISCUSSION**

The present study highlighted the importance of the eNOS-sGC pathway in compensatory renal hypertrophy after UNx. Compensatory renal hypertrophy was blunted in eNOS-deficient mice and, in marked contrast, was enhanced in EC-eNOSTG mice. The results of the in vitro study showed activation of the Akt-mTOR pathway in hPTECs by a NO donor and sGC stimulator, indicating the direct contribution of the eNOS-NO-sGC pathway to tubular hypertrophy in the absence of hemodynamic changes. In eNOS-deficient mice,
vascular resistance in many vascular beds (8, 33). The enzymatic activity of eNOS is stimulated by changes in the hydro-mechanical forces associated with pulsatile blood flow (24). Among such mechanical forces, shear stress is a major stimulus for endogenous NO production from the endothelium. NO plays an important role in the post-UNx renal hemodynamic response particularly in the early phase of this process (34). The hemodynamic adaptation after UNx includes increased renal blood flow coupled with reduced renal vascular resistance. Previous studies demonstrated that eNOS-induced enhanced production of NO plays a crucial role in this process (34). In addition to these hemodynamic adaptations, the present study demonstrated that NO derived from eNOS is also involved in the renal hypertrophy observed after UNx. It has been demonstrated that compensatory renal hypertrophy after UNx is mainly the result of hypertrophy and, in part, the result of hyperplasia of the renal cells, especially those in the proximal tubules, which account for the majority of kidney size increase after UNx. Post-UNx glomerular hyperfiltration is accompanied by increased tubular reabsorption (5a), and a previous study demonstrated that increased reabsorptive work-load in the tubules is not the primary cause of compensatory tubular hypertrophy. NO was produced by several isoforms of NOS, for example iNOS and nNOS. These isoforms may have an important role in compensatory renal hypertrophy. Actually eNOS-deficient mice had an increased nNOS mRNA level. Furthermore, Morishita et al. (18a) clarified the importance of several isoforms of NOS in physiological and pathophysiological status. But nNOS and iNOS mRNA levels were decreased 2-wk after nephrectomy. So these isoforms were perhaps not a major pathway in compensatory renal hypertrophy after nephrectomy.

In addition to the regulation of vascular tone, NO is involved in several physiological processes, including regulation of cellular proliferation, hypertrophy, and survival. Most NO effects are mediated by activation of sGC, generation of cGMP, and activation of PKG (12). While sGC functions as a NO sensor, it has been shown to promote angiogenesis in endothelial cells (26). In the present study, sGC activation by the selective sGC stimulator Bay 41–2272, restored compensatory renal hypertrophy after UNx in eNOS-deficient mice. Rapamycin treatment blunted the effect of sGC activation on renal hypertrophy, confirming that sGC activation is required for stimulation of protein synthesis associated with compensatory renal hypertrophy.

![Fig. 5](image)

**Fig. 5.** sGC stimulator increases PKG activity and phosphorylation of mTOR-associated protein. hPTECs were stimulated with or without 10 µM Bay 41–2272 for 30 min. A: PKG activity in hPTECs. Western blot analysis of phospho- and total Akt (B), phospho- and total 4EBP (C), and phospho- and total rps6 (D). Data are expressed relative to the value of Bay 41–2272 0 µM, which was used as a standard. E: TP-to-cell counts ratio was detected as an assessment of cell hypertrophy. Data are means ± SD. *P < 0.05.

![Fig. 6](image)

**Fig. 6.** Possible contribution of sGC activity to renal hypertrophy after Nx. NO does not only induce hemodynamic changes in the early phase of renal Nx but also activation of sGC, which results in tubular cell hypertrophy and maintenance of normal renal function.
The phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR pathway plays a role in renal hypertrophy in various pathological conditions. In diabetic animal models, PI3K and downstream signaling pathways are activated in the kidneys undergoing hypertension (4, 6). Previous studies indicated that the mTOR pathway also plays a role in the initiation of compensatory renal hypertrophy. Chen et al. (3) studied the regulation of mRNA translation in compensatory growth after UNx. Phosphorylation of both RPS6 and 4E-BP1 was abolished by administration of rapamycin, which also significantly inhibited the extent of renal hypertrophy. In addition, they demonstrated that deletion of S6 kinase 1 inhibited renal hypertrophy following either UNx or induction of diabetes. These data indicate that mTOR activation is a central event in compensatory renal growth (3). Our in vivo data add further support to this conclusion; our results showed that the mTOR inhibitor rapamycin cancelled the effects of the sGC stimulator on compensatory renal hypertrophy in eNOS-deficient mice. These findings also suggest that the sGC stimulator was modulated via activation of the mTOR pathway.

The underlying mechanism(s) leading to activation of mTOR signaling pathway remains unclear in post-UNx compensatory renal hypertrophy. The present study indicates that NO derived from renal eNOS plays an important role in this process. GSNO and Bay 41–2272 activated mTOR-associated protein in vitro through phosphorylation of Akt. Several lines of evidence indicate that PKG activates PI3K/Akt kinase pathway in vascular smooth muscle cells and endothelial cells (1, 11, 15). Wolfsgruber et al. (35) reported that a PKG-induced cGMP analog enhanced PI3K/Akt kinase signaling and proliferation and increased the levels of vascular cell adhesion molecule. Kawasaki et al. (13) also reported that NO itself directly activates PI3K/Akt signaling via the cGMP/PKG pathway, leading to endothelial cell growth and migration. Our data appear to show that PKG activation of hPTECs leads to NO-induced phosphorylation of Akt, suggesting that NO-sGC directly stimulates cellular hypertrophy via PKG activation without hemodynamic changes in human tubular cells.

Figure 6 shows the role of the eNOS-NO pathway in compensatory renal hypertrophy after UNx, highlighting the role of eNOS as an initial factor. UNx induces early hemodynamic changes such as increased shear stress in the kidney, which enhances eNOS activity. Shear stress elicits the phosphorylation of eNOS on tyrosine and serine residues and activates its enzymatic activity. The steady laminar shear stress increases the expression of eNOS mRNA and NO formation (18). NO production by eNOS does not only increase renal blood flow but also stimulates sGC activity in tubular cells. Activation of the sGC-PKG pathway stimulates mTOR pathway including the phosphorylation of rps6 kinase through Akt activation. In our in vitro study, it appears that activation of NO and sGC stimulated mTOR through Akt phosphorylation.

Clinical and experimental studies have demonstrated that endothelial dysfunction is associated with a decline in renal function. In a large group of hypertensive patients with normal or mild impairment of renal function, Perticone et al. (25) reported that endothelial dysfunction can be used to predict subsequent decline in the glomerular filtration rate. Aging is also associated with endothelial dysfunction as assessed by flow-mediated dilatation (16). Endothelial dysfunction in such conditions is speculated to trigger decompensatory renal hyper-

pertrophy. Interestingly, analysis of the compensatory renal hypertrophy after UNx in rats and humans has shown an inverse correlation between age and the compensatory increase in GFR, indicating impaired adaptation with age (10).

The present results indicate that sGC stimulation could restore the impaired adaptation to nephron reduction in eNOS-deficient mice. The therapeutic potential of drugs that increase cGMP levels has been confirmed by the clinical success of NO-generating drugs in the treatment of angina pectoris and congestive heart failure and, more recently, by the use of phosphodiesterase (PDE) inhibitors in the treatment of erectile dysfunction and pulmonary hypertension (7). Rodriguez-Iturbe et al. (27) reported that early treatment with the PDE5 inhibitor sildenafil prevented hypertension and slowed the progression of renal injury, although others have shown that delayed sildenafil treatment failed to improve proteinurea and kidney injury.

In conclusion, our results have highlighted the importance of the eNOS-NO-PKG in compensatory renal hypertrophy after UNx. NO induced both renal hemodynamic changes as well as directly stimulated sGC activation in tubular cells. Endothelial dysfunction promotes the switching from compensatory renal hypertrophy to progressive kidney injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.N., M.S., K.M.C., T.S., and N.K. conception and design of research; H.N. and K.M.C. performed experiments; H.N., M.S., K.M.C., T.S., and N.K. analyzed data; H.N. and K.M.C. interpreted results of experiments; H.N. and N.K. prepared figures; H.N., M.S., K.M.C., T.S., and N.K. drafted manuscript; H.N., M.S., K.M.C., and N.K. edited and revised manuscript; H.N. and N.K. approved final version of manuscript.

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