Transient nitric oxide reduction induces permanent cardiac systolic dysfunction and worsens kidney damage in rats with chronic kidney disease

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1Department of Nephrology, University Medical Center Utrecht, Utrecht and 2Department of Cardiology, Medical Center Utrecht, Utrecht, the Netherlands; 3Department of Nephrology and Immunology, University of Alberta, Edmonton, Alberta, Canada; 4Department of Pathology, University Medical Center Utrecht, Utrecht, the Netherlands; and 5Department of Nephrology, Meander Medical Center, Amersfoort, Netherlands

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Bongartz LG, Braam B, Verhaar MC, Cramer MJ, Goldschmeding R, Gaillard CA, Doevendans PA, Joles JA. Transient nitric oxide reduction induces permanent cardiac systolic dysfunction and worsens kidney damage in rats with chronic kidney disease. Am J Physiol Regul Integr Comp Physiol 298: R815–R823, 2010. First published December 23, 2009; doi:10.1152/ajpregu.00727.2009.—Left ventricular systolic dysfunction (LVSD) in patients with chronic kidney disease (CKD) is associated with poorer prognosis. Because patients with CKD often exhibit progressively decreased nitric oxide (NO) availability and inhibition of NO production can reduce cardiac output, we hypothesized that loss of NO availability in CKD contributes to pathogenesis of LVSD. Subtotally nephrectomized (SNX) rats were treated with a low dose of the NO synthase inhibitor Nω-nitro-L-arginine (L-NNA; 20 mg/l water; SNX+L-NNA) and compared with relevant control groups. To study permanent changes separate from hemodynamic effects, L-NNA was stopped after week 8 and rats were followed up to week 15, until blood pressure was similar in SNX+L-NNA and SNX groups. To study effects of NO depletion alone, a control group with high-dose L-NNA (L-NNA-High; 100 mg/l) was included. Mild systolic dysfunction developed at week 13 after SNX. In SNX+L-NNA, systolic function decreased by almost 50% already from week 4 onward. To distinguish combined effects of CKD and L-NNA, systolic function decreased by approximately 80% compared with relevant control groups by 10 weeks. LVSD was not as severe as in SNX+L-NNA, and renal function was reduced but less reduced than in SNX+L-NNA. Proteinuria increased with rats with SNX-1L-NNA, and glomerulosclerosis and cardiac fibrosis were worsened. We conclude that SNX+L-NNA induced accelerated and permanent LVSD that was functionally and structurally different from CKD or NO depletion alone. Availability of NO appears to play a pivotal role in maintaining cardiac function in CKD.

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PROGRESSION OF CHRONIC KIDNEY disease (CKD) coincides with increased incidence of cardiovascular disease (15), and left ventricular (LV) systolic dysfunction is one of the most important risk factors for development of heart failure and mortality (8, 16, 31). We and others (7, 17, 35) designated the coexistence of kidney and heart dysfunction as the (Severe) Cardiorenal Syndrome. As patients are far more likely to die of cardiovascular disease during the progression of CKD than to reach end-stage renal disease and dialysis (15), it is important to identify pathogenic factors that drive this increased risk. We proposed the Cardiorenal Connection as the putative pathophysiological mechanism (7) and further postulated that the balance between nitric oxide (NO) and reactive oxygen species is a key modulator of the other cardiorenal connectors (19).

Subtotal (5/6th) nephrectomy (SNX) in rats is one of the most widely used models to study the cardiac sequelae of CKD. However, measures of in vivo cardiac systolic function appear to be unchanged during study periods up to 8 wk (25, 34). In humans, the most common causes of CKD are hypertension, diabetes, and/or aging, which are associated with reduced NO availability. Indeed, CKD is accompanied by a long-standing and progressive decrease in NO availability (6, 44). Furthermore, inhibition of NO synthase (NOS) causes hypertension and cardiovascular damage and can functionally decrease systolic function and cardiac output in rats, dogs, and humans (3, 13, 26, 30, 39). We therefore aimed to explore the interaction between experimental CKD and chronically diminished NO availability on development of systolic dysfunction. We hypothesized that depletion of NO availability during progression of experimental CKD would cause cardiac systolic dysfunction and accelerate the development of cardiorenal failure. We studied this by treating SNX rats on a high-salt diet with low-dose NOS inhibition up to 8 wk after surgery (SNX+L-NNA) and compared in vivo heart and kidney function and whole body NO production with that in relevant control groups also on high salt. To distinguish combined effects of CKD and L-NNA on cardiorenal variables from those of systemic NO depletion per se, we also included a control group of normal rats treated with high-dose L-NNA designed to achieve similar levels of hypertension and NO depletion as in SNX+L-NNA.

Furthermore, while hemodynamic effects may reverse after discontinuation of chronic NOS inhibition, the resultant underlying cardiovascular damage can negatively affect renal function in the long term (12, 14). Therefore, we studied whether NO inhibition had caused persistent changes and studied cardiorenal functional variables not only during L-NNA treatment, but also after discontinuation of treatment, followed by evaluation of end organ damage upon termination.

METHODS

The study protocol was approved by the Ethical Committee on Animal Experiments of the University of Utrecht, Utrecht, The Netherlands, and conformed to Dutch law on Laboratory Animal Experiments. Male inbred Lewis rats, 180–200 g, were purchased from Charles River, Germany, and housed in a climate-controlled facility with a 12:12-h light-dark cycle.
Study Set-Up and Experimental Groups

Rats were divided into five groups with similar initial body weight: 1) control: sham-operated; 2) SNX: subtotal nephrectomy; 3) L-NNA-Low: sham-operated + 20 mg/L L-arginine (L-NNA; Sigma-Aldrich, St. Louis, MO) in drinking water; 4) L-NNA-High: sham-operated + 100 mg/L L-NNA in drinking water; 5) SNX + L-NNA: SNX + 20 mg/L L-NNA in drinking water (Fig. 1). The doses for L-NNA were determined in pilot experiments. The dose of 20 mg/L was chosen because it caused an increase in tail cuff systolic blood pressure (SBP; see below) up to 200 mmHg during L-NNA-treatment. The dose for L-NNA-High was chosen to achieve similar levels of hypertension and NO depletion as SNX + L-NNA. Animals in the L-NNA-Low, L-NNA-High, and SNX + L-NNA groups were pretreated with L-NNA during 2 wk before surgery. Control and SNX rats received normal water. All groups were fed standard pelleted rodent chow (CRM-E; Special Diet Services, Witham, Essex, UK). Baseline measurements of in vivo cardiac and renal function were performed to document dose-dependent effects of L-NNA before surgery. Starting in week 1, we then performed a two-stage SNX or the sham procedure. In short, the right kidney was first removed, and 1 wk later the poles of the left kidney were cut off, equalizing approximately two-thirds of the weight of the previously removed kidney. In sham-operated rats, the kidneys were only decapsulated. After 1 wk of recovery, rats again received L-NNA or normal water according to group, and all rats were fed standard powdered chow (CRM-FG; Special Diet Services) supplemented with 6% NaCl to induce acceleration of development of CKD. Although Szabo et al. (40) found that Lewis rats developed only mild CKD after SNX (by ablation/infarction), others have shown that ablation/infarction SNX in Lewis rats caused chronic stable kidney disease up to 15 wk with progressive proteinuria from 4 wk onward and a two- to threefold increase in serum creatinine levels (42). In pilot studies, we determined that SNX by surgical resection combined with 6% NaCl feed induced stable CKD after 6–8 wk, comparable to that described by Vercauteren et al. (42). We performed in vivo measurements of renal and cardiac function in weeks 4 and 8 after SNX. All L-NNA-treatments were then stopped at the end of week 8 in the respective groups. After a 3-wk washout period, cardiac and renal function was reevaluated every 2 wk up to week 15 (Fig. 1) until tail cuff SBP (see below) in SNX + L-NNA had decreased to the level of SNX alone.

Systolic Blood Pressure and 24-h Urine Samples

Systolic blood pressure (SBP) was measured by the tail cuff method as described previously (27). Directly after each SBP measurement, rats were placed in metabolism cages without food for 24 h, but with free access to water with 2% glucose, supplemented with L-NNA as appropriate, for determination of urinary protein measured with Coomassie blue. Urine was collected on 1 ml of antibiotic/antimycotic solution (cat. no. A5955; Sigma, St. Louis, MO) and stored at −80°C. Urinary excretion of stable NO metabolites NO$_2^-$/NO$_3^-$ was determined by fluorometric quantification of nitrite content (1). The rats were fasted during the 24-h collection period to minimize the effect of dietary protein intake. Thus, analysis of the 24-h NO excretion can provide a solid estimate of whole body NO production during 24 h (4). Creatinine clearance was calculated by the standard formula. Urinary sodium content was determined by flame photometry.

Echocardiography

Transcatheter echocardiography was performed with a digital ultrasound machine (model Sonos 5500, Philips Research, Eindhoven, The Netherlands) and a 15-MHz linear array transducer (Hewlett Packard, Palo Alto, CA). Animals were anesthetized with isoflurane and placed in a supine position on a warming pad and a three-lead ECG system was connected to the paws. Anesthesia was adjusted to the lowest possible level to maintain physiological heart rates. Two-dimensional B-mode cine-loops with continuous ECG-registration were recorded in the parasternal long-axis and the midpapillary short-axis views. Typical study duration was 15 min. We coded the acquisitions, and results were decoded after analysis. The recordings were analyzed off-line using the software present on the system, and the variables were measured in at least three heartbeats at end diastole and corresponding end systole. The investigator performing the analyses was blinded to treatment group.

Details on Calculations

Long axis. LV area (LVA) was measured by tracing the endocardial border; LV length (LVL) was measured from the apical trace border to the middle of the trace border on the LV outflow tract. LV volume was calculated with the ellipsoid area-length method: LV volume (ml) = [8*(LVA)$^2$/3 * LVL] at end diastole [end-diastolic volume (EDV)] and end systole [end-systolic volume (ESV)] (45). Calculated variables: stroke volume (SV; ml) = EDV – ESV; ejection fraction = (EDV – ESV)/ESV.

Short axis. The endocardial border was traced at end diastole (LVEDa) and end systole (LVESA) at the midpapillary level. Fractional area change of the LV (LV-FAC) was calculated with the formula: LV-FAC (%) = [(LVEDa-LVESA)/LVEDa] × 100%.

Plasma Parameters

After echocardiography, a blood sample (500 μl) was collected from the tail vein. Plasma was separated, and levels of urea (blood urea nitrogen) and creatinine were measured. Urea was determined by DiaSys Urea CT FS (DiaSys Diagnostic Systems, Holzheim, Germany) and creatinine was enzymatically determined with Creatinine F l-Type R1 and R2 (Wako Chemicals, Neuss, Germany).

Organ Weights and Histology

After exsanguination under anesthesia, we harvested the organs and weighed them. Kidneys were cut transversely, fixed in formalin, and embedded in paraffin. Glomerulosclerosis and tubulointerstitial damage were scored on periodic acid Schiff-stained kidney sections in a blinded manner as described previously (20–21).

After fixation in formalin, the heart was cut in three transverse sections and embedded in paraffin. Cardiomyocyte circumference was measured on periodic acid Schiff-stained myocardial slices in sections with transversely cut myocardial fibers by tracing the cellular border on photomicrographs of at least 50 different cardiomyocytes with a computer-assisted image analysis system (OptiMas, Houston, TX) in a blinded manner.
Digital photomicrographs of transverse sections of the heart stained with Sirius Red were taken to measure collagen content of the heart. The extent of cardiac fibrotic patches was scored in a blinded manner on joined digital images acquired at ×20 magnification using ImageJ software (33). Images were converted to an RGB stack, and collagen area fraction was measured on the green channel where the red-stained areas appear black. The percentage of the collagen area was calculated by dividing the Sirius Red-stained area by the total LV tissue area.

Perivascular collagen was analyzed by dividing the area of collagen around the vessel by total vessel area using ImageJ software on digital images acquired at ×200 magnification, visualized with circular polarized light.

Data Analysis

Data are shown as means ± SE. Data were analyzed and graphed using SigmaPlot 11.0 (Systat Software, San Jose, CA). One-way ANOVA with the Student-Newman-Keuls post hoc test was done per time point across all groups, and one-way repeated-measures ANOVA with Student-Newman-Keuls post hoc test across all time points within each group. Data that was not normally distributed was log transformed or ranked to achieve normality. Statistical significance was reached with $P$ values $< 0.05$.

RESULTS

Systolic Blood Pressure

Low-dose $\text{l-NNA}$ in rats without SNX caused mild hypertension. Mild hypertension was also present in SNX rats and treatment with low-dose $\text{l-NNA}$ in SNX+$\text{l-NNA}$ induced an increase in SBP up to levels of 200 mmHg (Fig. 2). Thus, the effect of low-dose $\text{l-NNA}$ and SNX on blood pressure appeared to be additive. In sham-operated rats, treatment with high-dose $\text{l-NNA}$ ($\text{l-NNA-High}$ group) led to a rise in blood pressure to similar levels as observed in SNX+$\text{l-NNA}$ rats during (low-dose) $\text{l-NNA}$-treatment. After cessation of $\text{l-NNA}$ treatment, SBP quickly normalized to pretreatment levels in $\text{l-NNA-Low}$ and $\text{l-NNA-High}$ groups. In contrast, in SNX+$\text{l-NNA}$, SBP tapered off slowly to the level of SNX.

Urinary NO Metabolites

Urinary NO metabolite (NOx) excretion, as a measure for whole body NO production, was mildly ($P = 0.06$) reduced in both SNX and $\text{l-NNA-Low}$ vs. control at 8 wk, but markedly depressed in $\text{l-NNA-High}$ and SNX+$\text{l-NNA}$ to similar low levels (Table 1). At week 15, 7 wk after stopping $\text{l-NNA}$, levels of urinary NOx in $\text{l-NNA-Low}$ and $\text{l-NNA-High}$, as well as in SNX, had increased to levels similar to control. This is in contrast to what we observed in SNX+$\text{l-NNA}$, where NO production remained significantly depressed compared with all other groups.

Echocardiography

Figure 3 shows the temporal changes of systolic function, expressed as LV-FAC in all groups. At the baseline measurement, after pretreatment in relevant groups before surgery (Fig. 1), there was a clear dose-dependent effect of $\text{l-NNA}$ on systolic function. In SNX, LV-FAC was similar to control up to week 8. Cardiac function was already severely impaired in SNX+$\text{l-NNA}$ at week 4, with LV-FAC dropping by almost 50% from baseline levels. In this group, we found an increase in ESV at week 8 vs. SNX, $\text{l-NNA-Low}$, and $\text{l-NNA-High}$ (Fig. 4). This resulted in a decreased ejection fraction and SV, despite the fact that EDV was also slightly larger in SNX+$\text{l-NNA}$ vs. SNX. Systolic dysfunction was also seen in $\text{l-NNA-High}$, but this was not as pronounced as in SNX+$\text{l-NNA}$ and was almost completely restored after stopping NOS inhibition. Surprisingly, left ventricular systolic dysfunction (LVSD) did not recover in SNX+$\text{l-NNA}$ after cessation of $\text{l-NNA}$.

At week 15, ejection fraction and LV-FAC were both mildly depressed in SNX vs. control, but only ejection fraction was significantly lower at week 15 in SNX vs. control (Fig. 4). On the other hand, SV was similar because of significant LV dilatation. Levels of LV-FAC of both control and SNX were significantly lower after week 8 compared with their baseline levels. Systolic function was still markedly low in SNX+$\text{l-NNA}$ at the end of the study with an increase in ESV and SV. Even when levels of LV-FAC of SNX+$\text{l-NNA}$ rats after week 8 were corrected for the decline in SNX rats compared with control, it was still significantly worse than SNX alone. Heart rate (Fig. 4E) was significantly lower in SNX rats compared with controls at week 15. However, because SV was slightly larger, calculated cardiac output was not different ($125 \pm 14$ ml/min vs. $130 \pm 8$ ml/min in SNX vs. control, respectively). Heart rate was reduced by high-dose $\text{l-NNA}$ at week 8, but not at week 15. There were no significant differences in heart rate between SNX and SNX+$\text{l-NNA}$.

Renal Variables

Treatment with $\text{l-NNA-Low}$ and $\text{l-NNA-High}$ did not affect renal function throughout the whole study period. Natriuresis levels were not significantly different between groups at week 8 and week 15. It should be noted that these values were obtained under fasting conditions. SNX induced CKD and resulted in raised plasma urea, plasma creatinine, and progressive proteinuria (Fig. 5, Table 1, and supplementary figure available online at the Am J Physiol Regul Integr Comp Physiol website). Calculated creatinine clearance in SNX was one-third of control at week 8 ($P < 0.001$) and declined further at week 15 ($P < 0.001$). At week 4, there were no significant differences between SNX and SNX+$\text{l-NNA}$ in either plasma urea (12 ± 1 mmol/l vs. 13 ± 1 mmol/l, respectively) or plasma creatinine (66 ± 6 μmol/l vs. 76 ± 5 μmol/l, respectively). Plasma urea was significantly higher in SNX+$\text{l-NNA}$ com-
Table 1. Kidney functional data at 8 wk and 15 wk

<table>
<thead>
<tr>
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<th>8 wk</th>
<th>15 wk</th>
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<tr>
<td></td>
<td>Control</td>
<td>SNX</td>
</tr>
<tr>
<td>No. rats</td>
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<td>8</td>
</tr>
<tr>
<td>Plasma urea, mmol/l</td>
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<td>0.4</td>
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<tr>
<td>Creatinine clearance, ml/min/100 g body wt</td>
<td>1.0</td>
<td>2.5</td>
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<tr>
<td>Urinary NOx, mol/24 h</td>
<td>0.13</td>
<td>0.89</td>
</tr>
<tr>
<td>Natriuresis, mol/24 h</td>
<td>36</td>
<td>617*</td>
</tr>
</tbody>
</table>

In all L-NNA-treated groups, L-NNA was stopped after 8 wk. Values are means ± SE. Control, sham operated; SNX, subtotally nephrectomized; L-NNA, N-nitro-L-arginine; L-NNA-Low, sham + 10 mg/l L-NNA; L-NNA-High, sham + 100 mg/l L-NNA; NOx, nitric oxide metabolites. *P < 0.05 vs. control; †P < 0.05 vs. SNX; ‡P < 0.05 vs. L-NNA-Low; §P < 0.05 vs. L-NNA-High. For clarity, different levels of significance are not indicated.

Fig. 3. Cardiac systolic function expressed as left ventricular fractional area change (LV-FAC, %) by echocardiography in the midpapillary short-axis view. Data are means ± SE. Symbols as in Fig. 2.

Mortality

Mortality in SNX + L-NNA was 30% (9 out of the initial 30 animals) by week 8. After stopping L-NNA, mortality continued to increase to 40% (12/30 animals) by week 11 and to 53% (16/30 animals) by week 15. Half of the mortality was spontaneous, and cause of death was undetermined. All other rats were euthanized because of severe morbidity. This consisted mainly of cachexia and dyspnea related to end-stage cardiac renal failure with visibly enlarged atria and thoracic edema. Others exhibited neurological deficits due to brain or spinal hemorrhage, a well-known side effect of long-term NOS inhibition (5). No morbidity or mortality occurred in any of the other groups.

Terminal Biometric Data

At the end of the study, both hematocrit and body weight were slightly, but significantly, lower in all rats with CKD compared with the other groups (Table 2). The left kidney remnant was hypertrophied to a larger degree in SNX than in SNX + L-NNA (Table 2). Terminal LV weight was also higher in all CKD rats compared with the other groups. Both right ventricle weight and wet lung weight were increased in SNX vs. control, and wet lung weight was slightly worsened by SNX + L-NNA (borderline significant).
Fig. 4. Echocardiographic variables derived from the long-axis view in all groups at 8 and 15 wk. End-diastolic volume (ml) (A); end-systolic volume (ml) (B); stroke volume (ml) (C); ejection fraction (D); and heart rate (E). Means ± SE. Symbols as in Fig. 2.
Histology

Histological damage is summarized in Table 3. SNX-induced glomerulosclerosis and tubulointerstitial injury. The number of severely sclerotic glomeruli was twofold increased by SNX+1-NNA. Parallel to the increase in LV mass, the cardiomyocyte area was higher in SNX compared with controls (P < 0.001), but this was not further aggravated in SNX+1-NNA. Cardiomyocyte hypertrophy was also observed in the 1-NNA-High group (P < 0.001 vs. control), despite the absence of increased LV weight (Table 2). SNX induced patchy fibrosis in the heart with increased collagen area fraction, which was worsened in SNX+1-NNA. High-dose NOS inhibition alone (1-NNA-High) also caused a moderate degree of fibrosis. Increased perivascular collagen was apparent in SNX, SNX+1-NNA, and 1-NNA-High groups, but not in the 1-NNA-Low group.

DISCUSSION

The main finding of this study is that minimal NOS inhibition in the context of renal failure (SNX+1-NNA) was sufficient to cause marked and persistent LV systolic dysfunction and worsened kidney damage. This occurred in association with a more profound and sustained depletion of whole body NO production than that observed with SNX or low-dose 1-NNA alone. Proteinuria and glomerulosclerosis, as well as cardiac fibrosis were more severe in SNX+1-NNA rats compared with rats with SNX alone. Cardiorenal failure was also worse in SNX+1-NNA than in sham-operated rats treated with a high dose of 1-NNA, despite similar levels of hypertension and NO depletion. The combination of SNX and low-dose 1-NNA induced combined cardiorenal failure that was functionally and structurally different from that induced by either CKD or NO depletion alone (as in 1-NNA-High).

Cardiac Changes

Our study suggests an important role for NO availability for maintenance of cardiac systolic function in CKD. Measures of in vivo systolic function generally remain preserved or even increase in rats with CKD with study periods up to 8 wk (25, 29, 34). Reddy et al. (34) argued that extending the period of CKD after SNX might induce a more progressive decline in cardiac function. We found only a slight decrease in systolic function in prolonged CKD up to 15 wk, and SV was not different from controls.

Addition of low-dose NOS inhibition during development of CKD in rats was sufficient to cause a striking decrease in LV systolic function. The cardiac dysfunction in SNX+1-NNA was due to an increase in ESV (Fig. 4). The difference in ESV at week 8 was much larger in SNX+1-NNA vs. SNX than in 1-NNA-High vs. control, while SBP was comparable between SNX+1-NNA and 1-NNA-High (203 ± 3 mmHg vs. 197 ± 7, respectively). Furthermore, LVSD was worse in SNX+1-NNA compared with 1-NNA-High. Levels of SBP were also similar in SNX+1-NNA and SNX at the end of the study, while cardiac systolic function was significantly more impaired in the former. It has been shown before that NOS inhibitors reduce cardiac output to a larger degree than equal pressor doses of vasoconstrictors (26). In acute NOS inhibition in dogs (26), as well as chronic NOS inhibition in rats (18), a decrease in cardiac output was observed without an effect on coronary blood flow. This suggests that negative inotropic effects of NOS inhibition could also play a role. However, total blood pressure load in SNX+1-NNA over the course of the whole study was likely higher than in rats with SNX alone, which might have played a role in the development of more cardiac fibrosis.

End-diastolic volume was slightly larger in SNX+1-NNA at week 8, which could be secondary to increased volume overload or compensation for loss of systolic ejection. Systolic function can also be affected by changes in preload, but both EDV and hematocrit were not different in SNX+1-NNA vs. SNX at the end of the study. On the other hand, the increased cardiac fibrosis in SNX+1-NNA may have hampered diastolic filling, leading to lower SVs. Constitutive NOS isoforms have diverse autocrine and/or paracrine effects on cardiomyocyte function (reviewed in Ref. 36). For example, neuronal NOS-

Table 2. Terminal biometric data

<table>
<thead>
<tr>
<th>No. rats</th>
<th>Control</th>
<th>SNX</th>
<th>1-NNA-Low</th>
<th>1-NNA-High</th>
<th>SNX+1-NNA</th>
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</thead>
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<tr>
<td>Hematocrit</td>
<td>0.49 ± 0.01</td>
<td>0.43 ± 0.01*</td>
<td>0.46 ± 0.00</td>
<td>0.48 ± 0.01</td>
<td>0.44 ± 0.01‡§</td>
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<tr>
<td>Body wt, g</td>
<td>426 ± 8</td>
<td>371 ± 17*</td>
<td>433 ± 9</td>
<td>425 ± 7</td>
<td>362 ± 7§</td>
</tr>
<tr>
<td>LK wt/kg body wt, g</td>
<td>3.34 ± 0.09</td>
<td>4.15 ± 0.12*</td>
<td>3.59 ± 0.08</td>
<td>3.62 ± 0.06</td>
<td>3.80 ± 0.09†</td>
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<tr>
<td>LV wt/kg body wt, g</td>
<td>1.93 ± 0.02</td>
<td>3.47 ± 0.21*</td>
<td>1.89 ± 0.02</td>
<td>2.07 ± 0.05</td>
<td>3.30 ± 0.06§</td>
</tr>
<tr>
<td>RV wt/kg body wt, g</td>
<td>0.49 ± 0.03</td>
<td>0.67 ± 0.06*</td>
<td>0.44 ± 0.03</td>
<td>0.48 ± 0.03</td>
<td>0.60 ± 0.05</td>
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<tr>
<td>Wet lung wt/kg body wt, g</td>
<td>3.09 ± 0.09</td>
<td>3.91 ± 0.28*</td>
<td>3.09 ± 0.04</td>
<td>3.28 ± 0.07</td>
<td>5.31 ± 0.55§</td>
</tr>
</tbody>
</table>

Values are means ± SE. LK, left kidney; LV, left ventricle; RV, right ventricle. *P < 0.05 vs. control; †P < 0.05 vs. SNX; ‡P < 0.05 vs. 1-NNA-Low; §P < 0.05 vs. 1-NNA-High. For clarity, different levels of significance are not indicated.
Commercially derived NO appears to modulate excitation-contraction coupling of the cardiomyocyte by regulating calcium fluxes. Isolated cardiomyocytes from uremic rats showed reduced sarcoplasmic reticulum calcium ATPase-2a activity and disturbed calcium cycling (24), and inhibition of NO production in SNX+L-NNA might have worsened these effects. Finally, the absence of meaningful differences in heart rate between groups suggests that cardiac output followed changes in SV. The level of anesthesia during echocardiography was specifically adjusted to the lowest possible level to avoid reductions in heart rate and blood pressure. The small, nonsignificant reduction in heart rate at week 8 in SNX+L-NNA compared with SNX alone means that cardiac function in the former group was even worse than estimated by SV and ejection fraction. More importantly, no compensatory increase in heart rate, which might have confounded the observed LVSD, was seen in any of the treated groups. The fact that we used a high-salt diet to accelerate progression of CKD in SNX rats might have affected the observed cardiac changes by altering fluid volume status or cardiac damage. However, all groups received the high-salt diet and natriuresis was not significantly different between groups.

Thus, a combination of functional and structural effects of the combined CKD and NO depletion likely caused the persistent systolic dysfunction. Our observations indicate that the experimental CKD made the heart more susceptible to the cardiodepressive effects of NO depletion. A CKD-specific effect on cardiac dysfunction is supported by clinical observations that LVSD and LV hypertrophy can reverse in a significant number of patients after renal transplantation, but the underlying mechanisms are incompletely understood [reviewed by Zolty et al. (47)].

Inhibition of NO production alone in L-NNA-Low and L-NNA-High caused dose-dependent hypertension and systolic dysfunction that reversed to control levels after stopping L-NNA, which is in accordance with previous findings (12-14, 18). Some mild permanent effects on cardiac remodeling and function were apparent in L-NNA-High at week 15, which are probably linked to the cardiomyocyte hypertrophy and mild fibrosis present in these hearts. Cardiomyocyte size was, however, similarly increased in the hypertrophied SNX and SNX+L-NNA hearts as in the non-hypertrophic L-NNA-High hearts. The different hypertrophic and fibrotic responses in SNX, SNX+L-NNA, and L-NNA-High are in agreement with previous observations that the LV hypertrophy and fibrosis observed in CKD appear to be partly independent of blood pressure load (28, 32, 38).

**Changes in NO Production**

Both SNX alone and L-NNA-Low alone exhibited a mild reduction in urinary NOx excretion compared with controls, while in SNX+L-NNA it was markedly decreased. This suggests that the separate interventions reduce whole body NO production via alternate pathways and have additive effects when combined. This also suggests that in SNX alone there was reserve capacity of NO production, which was blocked by addition of an exogenous NO synthase inhibitor. Rats with L-NNA-High also had a significantly lower NOx excretion at 8 wk compared with untreated controls, which was not statistically different from SNX+L-NNA. However, cardiac dysfunction was significantly worse in SNX+L-NNA. Thus, a similar reduction in NO availability has a stronger impact on LV systolic function in combination with CKD than either of these alone. The additive effects of low-dose NOS inhibition in the CKD rats were most likely not due to accumulation of L-NNA, because L-NNA is not excreted through the kidneys (41). Furthermore, it is highly likely that all L-NNA was fully converted into L-arginine, which is in accordance with previous findings (37) or to iNOS-derived NO related to injury. However, the latter is unlikely, considering that tubulointerstitial injury was not significantly different from SNX+L-NNA. Although salt intake can influence effects of NOS inhibition (46), the fact that there was no renal dysfunction and only minimal damage in L-NNA-Low and L-NNA-High excludes a major interaction.

Urinary NOx excretion was not reduced in SNX animals at week 15 compared with controls. This could either be due to increased NO production needed to maintain natriuresis (37) or to iNOS-derived NO related to injury. However, the latter is unlikely, considering that tubulointerstitial injury was not significantly increased in SNX+L-NNA vs. SNX. The high-salt diet may have slightly worsened hypertension in SNX, which can increase NO production due to higher shear-stress in resistance vessels.

Urinary NOx excretion was still significantly reduced at week 15 in SNX+L-NNA. The combination of CKD and temporary NOS inhibition apparently induced changes that persistently reduce net NO production. These might include decreased NOS enzyme density or activity, L-arginine deficiency, or increased levels of endogenous inhibitors like asymmetric dimethylarginine (2).
Renal Changes

Previously, Fujihara et al. (11) treated SNX rats with low-dose Nω-nitro-l-arginine (l-NNAME), a similar NOS inhibitor, up to 3 wk after SNX. They found increased hypertension and intraglomerular pressure, associated with more severe renal failure and kidney damage. Kang et al. (23) administered l-NNAME over a 4-wk period, starting 4 wk after SNX surgery, and found similar effects. Thus, NOS inhibition during both the initial and later stage of CKD development appears to worsen renal end points. In our study, renal failure was only mildly aggravated during l-NNA-treatment, as evidenced by significantly higher plasma urea and numerically higher plasma creatinine in SNX+l-NNA compared with SNX at week 8. However, these differences were no longer appreciable at week 15. Furthermore, creatinine clearance was not lower in SNX+l-NNA at week 8 and was even numerically higher at week 15 compared with SNX alone. In contrast, we observed consistently higher levels of proteinuria from week 8 onward in SNX+l-NNA compared with SNX alone. Inhibition of NOS can worsen proteinuria in rats with diabetic nephropathy with no effect on creatinine clearance (22). Thus, different disease states that are generally associated with a decrease in NO availability make the kidneys more sensitive to (further) reductions in NO, and the permanent reduction in NO availability seen in SNX+l-NNA may be the driving factor for the increased protein leakage, despite the fact that SBP had returned to levels of SNX at week 15.

Kidney function was not affected by either l-NNA-Low or l-NNA-High alone in sham-operated rats, and the high-dose group displayed only a minor degree of glomerulosclerosis. A similar high dose of 1-NNA has been shown to induce significant hypertension, proteinuria, and mortality in Wistar Kyoto rats (43). This suggests that the inbred Lewis rat, like the Wistar Furth rat (9), is more resistant to NO depletion. The fact that renal function in Lewis rats was not compromised in l-NNA-High allows more accurate separation of the effects of reduced vs. intact kidney function, combined with systemic NO depletion. The Wistar Furth rat was also more resistant to development of CKD after SNX, but this was greatly accelerated by subsequent treatment with low-dose NOS inhibition, concurrent with a high mortality rate (28%) (10). This increased mortality may well have been due to cardiac events.

We used a high-salt diet in our experiments. This was done to accelerate progression of CKD after SNX, because Lewis rats appear to be more resistant to SNX than other strains (40). Nevertheless, with this combination we could produce a model of chronic stable CKD similar to the model of Verscauteren et al. (42). All groups received the high-salt diet, and natrurexis was not significantly different between groups at week 8 and week 15 (Table 1), suggesting that all groups were in sodium balance. The absence of significant renal changes in the l-NNA-Low and l-NNA-High groups suggests that the interaction between the high-salt diet and NOS inhibition on renal function was minimal, but we cannot rule out that results may have been different in rats on normal salt intake.

At 4 wk post-SNX, the levels of plasma urea, plasma creatinine, and proteinuria were all similar in SNX+l-NNA compared with SNX, but systolic function was already severely compromised in SNX+l-NNA. Thus, the initial development of systolic dysfunction in SNX+l-NNA could not be attributed to more severe renal damage or dysfunction. Nevertheless, the persistence of LVSD at later stages might well relate to the worsened kidney damage that developed later on, as evidenced by increased proteinuria and more severe glomerulosclerosis in SNX+l-NNA.

Conclusions

Transient low-dose NOS inhibition during development of experimental CKD induced severe and permanent cardiac systolic dysfunction and persistent NO depletion in association with high mortality. The combination of SNX and temporary low-dose l-NNA caused end organ dysfunction and structural damage in heart and kidneys that was worse than that induced either by long-term CKD, or by temporary high-dose NOS inhibition alone, despite an equal degree of hypertension and NO depletion. Our results underscore the importance of adequate NO availability for maintenance of cardiorenal function in the face of reduced kidney function. Furthermore, the cardiorenal failure observed in the model of SNX+l-NNA might serve as a more accurate representation of advanced CKD in patients, associated with decreased NO availability and cardiac systolic dysfunction.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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