Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure

Hyun Ju Kim and Nosratola D. Vaziri

Division of Nephrology and Hypertension
University of California
Irvine, California

Running head: Defective Nrf2-mediated antioxidant response in CKD

Address all correspondence to:
N.D. Vaziri, M.D., MACP
University of California, Irvine Medical Center
Division of Nephrology and Hypertension
The City Tower, 4th floor
Orange, CA 92868
714-456-5142; 714-456-6034 (FAX)
ndvaziri@uci.edu
ABSTRACT

Oxidative stress and inflammation are constant features and major mediators of progression of chronic kidney disease (CKD). Nuclear factor E2-related factor-2 (Nrf2) confers protection against tissue injury by orchestrating antioxidant and detoxification responses to oxidative and electrophilic stress. While sources of oxidative stress and inflammation in the remnant kidney have been extensively characterized, the effect of CKD on Nrf2 activation and expression of its downstream gene products is unknown and was investigated. Subgroups of male Sprague-Dawley rats were subjected to 5/6 nephrectomy or sham operation and observed for 6 or 12 weeks. Kidneys were then harvested and Nrf2 activity and its downstream target gene products (antioxidant and phase II enzymes) were assessed. In addition, key factors involved in promoting inflammation and oxidative stress were studied. In confirmation of earlier studies, rats with chronic renal failure exhibited increased lipid peroxidation, glutathione depletion, NFκB activation, mononuclear cell infiltration, and upregulation of MCP-1, NAD(P)H oxidase, cyclooxygenase-2, and 12-lipoxygenase in the remnant kidney pointing to oxidative stress and inflammation. Despite severe oxidative stress and inflammation, remnant kidney tissue Nrf2 activity (nuclear translocation) was mildly reduced at 6 weeks and markedly reduced at 12 weeks whereas Nrf2 repressor, Keap1, was up-regulated and products of Nrf2 target genes (catalase, superoxide dismutase, glutathione peroxidase, heme oxygenase-1, NAD(P)H quinone oxidoreductase, glutamate-cysteine ligase) were reduced or unchanged at 6 weeks and significantly diminished at 12 weeks. Thus, oxidative stress and inflammation in the remnant kidney are compounded by conspicuous
impairment of Nrf2 activation and consequent downregulation of the antioxidant enzymes.

Key words: Chronic kidney disease, CKD progression, Anti-inflammatory factors, Antioxidant response, NAD(P)H oxidase, Superoxide dismutase, Glutathione, Lipid peroxidation
INTRODUCTION

Oxidative stress and its constant companion inflammation are common features of chronic kidney disease (CKD) and major mediators of its cardiovascular and numerous other complications (10, 11, 37, 40, 42, 44, 48). In addition oxidative stress and inflammation play a critical part in progression of CKD (7, 24, 30, 31, 39, 44). Oxidative stress in CKD is caused by a combination of increased production of reactive oxygen species (ROS) (4, 38-40, 42, 44) and impaired antioxidant capacity (10, 11, 26, 34, 37, 41).

Increased generation of ROS leads to tissue injury and dysfunction by attacking, denaturing and modifying structural and functional molecules and by activating redox-sensitive transcription factors and signal transduction pathways. These events, in turn, promote necrosis, apoptosis, inflammation, fibrosis and other disorders. Redox systems including antioxidant enzymes and phase II detoxifying and antioxidant agents provide protection against ROS-induced tissue injury. Nuclear factor-erythroid-2-related factor 2 (Nrf2) plays a critical part in basal activity and coordinated induction of genes encoding numerous antioxidant and phase II detoxifying enzymes and related proteins such as catalase, superoxide dismutase (SOD), UDP-glucuronosyltransferase, NAD(P)H:quinone oxidoreductase-1, heme oxygenase-1, glutamate cysteine ligase, glutathione S-transferase, glutathione peroxidase and thioredoxin, among others (22). Nrf2 is held in the cytoplasm as an inactive complex bound to a repressor molecule known as Keap1 (Kelch-like ECH-associated protein 1) which facilitates its ubiquitination. Keap1 contains several reactive cysteine residues that serve as sensors of intra-cellular redox state. Oxidative or covalent modification of thiols in some of these cysteine residues results in dissociation of Nrf2
from Keap1 and its translocation to the nucleus. In the nucleus, Nrf2 binds to the regulatory sequences, termed antioxidant response elements (ARE) or electrophile response elements (EpRE), located in the promoter region of genes encoding the antioxidant and phase 2 detoxifying enzymes. This process is mediated by heterodimerization of Nrf2 with other transcription factors, such as small Maf, within the nucleus. It is of note that nuclear translocation of Nrf2 may also occur via phosphorylation of some of its threonine or serine residues by upstream kinases such as protein kinase C, mitogen-activated protein kinases, phosphatidylinositol-3-kinase/Akt, and casein kinase-2 (35).

The Nrf2-mediated regulation of cellular antioxidant and anti-inflammatory machinery plays an important role in defense against oxidative stress (22). In fact disruption of Nrf2 in mice diminishes or abrogates the induction of these antioxidant genes, indicating their Nrf2-dependent regulation. Moreover, Nrf2 gene ablation has been shown to cause a lupus-like autoimmune nephritis and exacerbate diabetes-induced inflammation, oxidative stress and renal injury in the experimental animals (46, 47).

While pathways involved in intra-renal ROS production and inflammation in experimental CKD have been widely explored (4, 7, 37-40, 42, 44), the effect of CKD on Nrf2-Keap1 system and its downstream gene products is unknown. The present study was undertaken to address this issue.

**MATERIAL AND METHODS**

**Animals** – Male Sprague Dawley rats, weighing 225-250 g, were purchased from Harlan Sprague Dawley Inc (Indianapolis, IN). They were housed in a climate-controlled and light-regulated facility with 12:12-h day-night cycles. The animals were fed regular rat
chow (Purina Mills, Brentwood, MO) and water ad libitum and randomly assigned to the chronic renal failure (CRF) and normal control groups. The animals assigned to the CRF group were subjected to 5/6 nephrectomy by surgical resection using a dorsal incision, as described previously (39). The animals assigned to the control group were subjected to sham operation. All surgical procedures were carried out under general anesthesia (Nembutal 50 mg/kg IP). Strict hemostasis and aseptic techniques were observed. Subgroups of rats were observed for 6 or 12 weeks at which time they were placed in metabolic cages for a 24-hour urine collection. They were then anesthetized (pentobarbital 50 mg/kg IP) and euthanized by exsanguinations using cardiac puncture. The kidneys were immediately removed, frozen in liquid nitrogen, and stored at -70°C until processed. All experiments were approved by the University of California, Irvine Institutional Committee for the Use and Care of Experimental Animals. Plasma glutathione level was measured by HPLC and serum creatinine, urea, total cholesterol, triglyceride concentrations, urinary protein excretion were measured as described in previous studies (39).

**Measurement of blood pressure** – Blood pressure was determined by tail plethysmography (CODA2, Kent Scientific Corporation, Torrington, CT). In brief, the conscious animal was placed in a restrainer and permitted to rest for 10 to 15 min. The cuff was then placed on the tail and was inflated and released several times to condition the animal to the procedure. After stabilization, blood pressure was measured three times, and the average of the values obtained was used.

**Thiobarbituric acid (TBA)-reactive substance (TBARS) levels** – Serum malondialdehyde (MDA) was measured by the method of Naito & Yamanaka (29). Renal TBARS was
assayed according to the method of Mihara & Uchiyama (25). Briefly, the tissue was homogenized with a 9-fold volume of ice-cold 0.9% NaCl solution. Mitochondria were prepared from kidney homogenates by differential centrifugation (800g and 12,000g at 4ºC for 15 min) according to the methods of Johnson and Lardy (15) and Jung and Pergande (16), with slight modifications. Each pellet was resuspended in preparation medium. A sample of homogenate or pellet suspension was mixed with 1% H₃PO₄ and 0.67% TBA, and boiled for 45 min. After cooling in ice water, the reaction mixture was extracted with n-BuOH. TBA-reactive substance was determined by measuring the absorbance at 532 nm. The value of TBA-reactive substance was expressed in nmol MDA/mg protein by a calibration curve constructed from standard MDA (0-100 nmol/ml) in 1,1,3,3-tetramethoxypropane. Protein level was evaluated by the method of Itzhaki and Gill (14) using bovine serum albumin as the standard.

**Preparation of kidney homogenates and nuclear extracts** — All solutions, tubes, and centrifuges were maintained at 0-4ºC. The nuclear extract was prepared as described previously (33). Briefly, 100 mg of kidney cortex was homogenized using a glass-Teflon homogenizer in 0.5 mL buffer A containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM PMSF, 1μM pepstatin, and 1 mM P-aminobenzamidine using a tissue homogenizer for 20 sec. Homogenates were kept on ice for 15 min and then 125 μL of a 10% Nonidet p40 (NP 40) solution was added and mixed for 15 sec, and the mixture was centrifuged for 2 min at 12,000 rpm. The supernatant containing cytosolic proteins was collected. The pelleted nuclei were washed once with 200 μL of buffer A plus 25 μL of 10% NP 40, centrifuged, then suspended in 50 μL of buffer B (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1
mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% (v/v) glycerol), mixed for 20 min, and centrifuged for 5 min at 12000 rpm. The supernatant containing nuclear proteins was stored at –80°C. The protein concentrations in tissue homogenates and nuclear extracts were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

**Western blot analyses** – Target proteins in the cytoplasmic and/or nuclear fractions of the kidney tissue were measured by Western blot analysis using the following antibodies:

- Rabbit antibodies against rat NF-κB p65, NF-κB p50, p22phox, NOX4, MCP-1, COX-2, Nrf2, Keap1, HO-1, NQO1, GCLC and GCLM antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).
- Antibodies against 12-lipoxygenase (12-LO) (Cayman chemical, Ann Arbor, MI), Cu,Zn-SOD and catalase (EMD Chemicals, Inc., Gibbstown, NJ), glutathione peroxidase (Gpx) and Mn-SOD (Millipore, Billerica, MA), phospho-IκB-α (Cell Signaling Technology, Inc., Denver, CO), gp91phox, p47phox and Rac1 (BD bioscience, San Jose, CA) were purchased from the cited sources.
- Antibodies to Histone H1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), β-actin (Sigma Inc., Saint Louis, Missouri) and GAPDH (Imgenex, San Diego, CA) were used for measurements of the housekeeping proteins for nuclear and cytosolic target proteins respectively.

Briefly, Aliquots containing 50 µg proteins were fractionated on 8% and 4 - 20% Tris-glycine gel (Novex Inc., San Diego, CA, USA) at 120 V for two hours, and transferred to Hybond-ECL membrane (Amersham Life Science Inc., Arlington Heights, IL). The membrane was incubated for one hour in blocking buffer (1 x TBS, 0.05 % Tween-20 and 5 % nonfat milk) and then overnight in the same buffer containing the given antibodies. The membrane was washed three times for 5 minutes in 1 x TBS,
0.05 % Tween-20 prior to two-hour incubation in a buffer (1 x TBS, 0.05 % Tween-20 and 3 % nonfat milk) containing horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG (Amersham Life Science Inc.) at 1:1000 dilution. The membrane was washed 4 times and developed by autoluminography using the ECL chemiluminescent agents (Amersham Life Science Inc.)

**Data Analysis** – Student t-test was used in statistical evaluation of the data which are shown as mean ± SD, unless otherwise specified. $P$ values less than 0.05 were considered significant.

**RESULTS**

**General Data** – The CRF group exhibited hypertension, proteinuria, increased plasma urea, creatinine, triglycerides and total cholesterol concentrations and marked remnant kidney hypertrophy (Table 1).

**Renal histology** - The CRF group exhibited significant glomerulosclerosis, tubulo-interstitial injury and heavy mononuclear cell infiltration in the remnant kidney (Figure 1).

**Markers of oxidative stress** – Data are shown in Figure 2. Compared to the control group the CRF group exhibited significant elevation of plasma MDA concentration. This was accompanied by a marked increase in TBARS in the renal tissue homogenate as well as mitochondrial fraction of the kidney in the CRF animals compared to those found in the control group. In confirmation of our earlier studies (42) plasma concentration of nitrotyrosine, a footprint of nitric oxide interaction with reactive oxygen species was markedly elevated in the CRF group (data not shown). In addition, plasma concentration of reduced glutathione (GSH) was markedly reduced and that of oxidized glutathione (GS-SG) was increased and the GSH/GS-SG ratio was markedly diminished in the CRF
animals. These findings point to heightened ROS-induced lipid peroxidation and nitric oxide and glutathione oxidation in the CRF animals.

**Oxidative and inflammatory pathways** – Data are illustrated in figures 3-5. Compared to the control group, the CRF group studied 12 weeks after renal ablation showed significant increase in protein abundance of the NADPH oxidase subunits; NOX4, gp91phox, p22phox, p47phox, and Rac1 in the remnant kidney. Similarly, MCP-1, 12-LO and COX-2 abundance were significantly increased in the remnant kidneys of the CRF groups compared with the corresponding values found in kidneys of the control animals. This was associated with a significant increase in phosphorylated IκB and elevated nuclear contents of p50 and p65 active subunits of NF-κB which is the master regulator of many pro-inflammatory and pro-fibrotic cytokine and other mediators. Examination of renal tissues obtained at 6 weeks after renal ablation revealed directionally similar but less intense changes in the above parameters (Figures 5 and 6).

**Nrf2/Keap1 pathway** – Data are shown in figures 7-10. Compared to the control group the CRF group showed a mild and insignificant reduction of nuclear Nrf2 abundance and a significant increase in cytoplasmic Keap1 abundance in the remnant renal tissue at 6 weeks. The CRF-induced reduction of nuclear Nrf2 abundance and elevation of cytoplasmic Keap1 abundance were more intense at 12 weeks. This was associated with significant reduction of renal tissue HO-1, GCLC, GCLM and NQO1, Catalase, GPX, Cu,Zn-SOD and Mn-SOD protein abundance in the remnant kidney at 12 weeks post 5/6 nephrectomy. Changes in the above parameters in the remnant kidney tissues at 6 weeks following renal ablation were less intense (Figures 5 and 6). Conspicuous failure of the Nrf2 activation at 6 weeks post renal ablation and marked reduction of its activity at 12
weeks despite upregulation of oxidative and inflammatory pathways point to
dysregulation of this vital defense mechanism in CRF.

**DISCUSSION**

Nrf2 is a redox-sensitive transcription factor which plays a vital role in protection against oxidant- and xenobiotic-induced cellular injury (9, 17, 23). Nrf2 regulates basal activity and coordinated induction of genes encoding antioxidant and phase II detoxifying enzymes including catalytic (GCLC) and modulator (GCLM) subunits of the rate-limiting enzyme in glutathione biosynthesis (glutamate-cysteine ligase), detoxification enzymes (GST, NQO1) and antioxidant enzymes (CAT, GPx, SOD, HO-1). Via activation of Nrf2 and consequent expression of the antioxidant and detoxifying enzymes, reactive oxygen species elicit a compensatory response aimed at mitigating the impact of oxidative stress and its constant companion, inflammation (2, 3, 9). In fact there is mounting evidence supporting the protective role of Nrf2-mediated pathway against oxidative stress and inflammation (Figure 9). For instance the inflammatory response to activation of NF-κB and consequent induction of COX-2, inducible nitric oxide synthase (iNOS), interleukin (IL)-6 and tumor necrosis factor (TNF)-α is more intense in Nrf2 knock out mice compared with the wild-type mice (3, 22). In addition, hyperglycemia results in more severe oxidative stress and renal injury in Nrf2-deficient than in the wild-type mice with streptozotocin-induced diabetes (46, 47). Conversely, activation of Nrf2 has been shown to confer protection against oxidized lipid/lipoprotein-induced damage in endothelial cells, vascular smooth muscle cells and macrophages (2, 3, 12, 20). In fact certain anti-inflammatory and antioxidant phytochemicals capable of activating Nrf2 signaling can enhance cellular defense against oxidative and electrophilic insults (36).
Oxidative stress and inflammation are constant features of advanced renal disease and play a major role in progressive deterioration of renal function and structure and the associated cardiovascular and numerous other complications of CKD. The CRF animals studied 12 weeks after 5/6 nephrectomy exhibited oxidative stress, inflammation, NF-κB activation and upregulation of COX-2, 12-LO and ROS-generating enzyme, NAD(P)H oxidase, in the remnant kidney. While pathways involved in intra-renal ROS production and inflammation in experimental CKD have been widely explored (4, 7, 37-40, 42-44), the effect of CKD on Nrf2-Keap1 system and its down stream gene products is unknown and was explored here.

Despite severe oxidative stress and inflammation which should have induced activation of Nrf2 and up-regulation of its down-stream gene products, the CRF group showed progressive reduction of nuclear Nrf2 content, signifying diminished activation of this transcription factor in remnant kidney. This was accompanied by significant down-regulation of the Nrf2 target gene products including the antioxidant enzymes (catalase, SOD, GPX and HO-1), glutamate-cysteine ligase (GCLC, GCLM), the key enzyme in glutathione synthesis, and the detoxifying enzyme, NQO1 at 12 weeks and a less severe decline or lack of expected rise at 6 weeks. This phenomenon points to the impaired ability of uremic animals to mount the biological response to the prevailing oxidative stress and inflammation and their destructive consequences in the remnant kidney.

The paradoxical reduction of Nrf2 activation in the face of severe oxidative stress and inflammation was accompanied by significant elevation of Keap1 abundance in the remnant kidneys of the CRF animals. Keap1 is a cysteine-rich protein which serves as a
redox sensor, an inhibitor of nuclear translocation of Nrf2 and a facilitator of its proteasomal degradation (13). Therefore, conspicuous lack of Nrf2 activation despite the prevailing oxidative stress in the remnant kidney shown in the present study must be, in part, due to increased Keap1 abundance. As noted above genetic disruption of Nrf2 has been shown to amplify severity of oxidative stress and inflammation and intensify tissue injury in Nrf2 knock out mice (46, 47). Likewise ischemic and nephrotoxic insults result in a much more severe acute kidney injury and dysfunction and higher mortality in Nrf2-deficient than in the wild-type mice and can be substantially ameliorated by administration of reduced glutathione or its precursor, N-acetyl cysteine (21). Therefore, acquired deficiency of the Nrf2 pathway shown for the first time here must contribute to the severity of oxidative stress and inflammation and progression of tissue damage in the remnant kidney in this model.

GSH is the most abundant endogenous antioxidant in eukaryotic cells and is a major player in regulation of the cellular redox state (45). GSH maintains cellular redox status and affects redox signaling, cell proliferation and cell death (9). GSH exerts potent antioxidant actions by directly scavenging ROS and by serving as the substrate in reactions catalyzed by a number of major antioxidant enzymes such as glutathione peroxidase. Selective inhibition of enzymes of the glutathione redox cycle heightens the susceptibility to ROS-mediated cell injury (8) and many pathologic conditions including atherosclerosis, diabetes, liver disease, myocardial infarction, and stroke are associated with elevated GSSG and diminished GSH levels (3, 5, 19, 28, 32). Expression of glutamate-cysteine ligase, the rate-limiting enzyme in glutathione synthesis, is regulated by Nrf2. The CRF rats employed in the present study showed significant elevation of
lipid peroxidation products and marked reductions of glutamate-cysteine ligase (GCLC and GCLM) and the GSH / GS-SG ratio (reflecting depressed cellular redox status) in the renal tissue.

In addition to regulating expression of GSH producing enzyme, Nrf2 regulates expression of various antioxidant/detoxifying enzymes utilizing GSH as their substrate. Chief among them are glutathione S-transferase which is involved in detoxification of short-chain aldehydes derived from lipid peroxidation and GPX which plays a critical role in reduction of hydroperoxides and lipid hydroperoxides. Normally, oxidized lipids induce Nrf2 activation, GSH production and expression of antioxidant enzymes including HO-1, and peroxiredoxin I (Prx I) as well as stress protein A170. These events serve as a compensatory response aimed at mitigating oxidative stress and preventing cellular injury (2, 5, 12). However, despite presence of large amounts of oxidized lipids, Nrf2 activity, GSH production capacity and related antioxidant enzymes were depressed in the remnant kidney, reflecting a maladaptive response. Thus reduction in GSH production capacity and down-regulation of the related enzymes in the remnant kidney can lead to accumulation of hydroperoxides and lipoperoxides, perpetuation of oxidative stress and inflammation and progression of kidney disease. In addition, presence of uncontained oxidized lipids and lipoproteins in the remnant kidney can lead to irrepressible lipid uptake by macrophages and resident cells via up-regulation of scavenger receptors leading to foam cell formation and lipotoxicity in the remnant kidney (18).

Oxidative stress in the remnant kidney of CRF animals was accompanied by activation of NF-κB, heavy infiltration of mononuclear leukocytes, and up-regulation of MCP-1, COX-2 and 12-LO representing intense inflammatory reaction. Reactive oxygen
species particularly hydroperoxides are potent activators of the redox-sensitive transcription factor, NF-κB, the general transcription factor for many pro-inflammatory cytokines and chemokines. Thus, inability to contain oxidative stress, occasioned by Nrf2 deficiency, must contribute to the NF-κB activation and inflammation in the remnant kidneys of the CRF animals. This assertion is consistent with previous observations which illustrated the anti-inflammatory function of Nrf2 (3, 22, 27, 36). In this context deficiency of HO-1, which is regulated by Nrf2 has been shown to accentuate glomerulonephritis (6) and cause a lupus nephritis-like disease (47). In contrast, upregulation of HO-1 via Nrf2 activation dampens NF-κB-dependent responses to pro-inflammatory cytokines (1) and Nrf2 gene transfer attenuates inflammation in the vessel wall (20). Thus impaired Nrf2 activity in the remnant kidneys of CRF animals is, in part, responsible for the prevailing inflammation and its adverse consequences in this model.

In conclusion, despite severe oxidative stress and inflammation, which should have induced Nrf2 activation and consequent upregulation of anti-oxidant and detoxifying enzymes, the remnant kidney in CRF animals exhibited paradoxical reduction of Nrf2 activation and its down-stream antioxidant molecules. Accordingly impaired Nrf2 activation shown for the first time here contributes to the severity of oxidative stress and inflammation and progression of tissue damage in the remnant kidney in this model. Further studies are planned to determine the effect of pharmacological activation of Nrf2 in this model.


Figure Legend

**Figure 1** - Representative photomicrographs of the renal tissue in a 5/6 nephrectomized (CRF) and a sham-operated control (CTL) rat. The remnant kidney in the CRF animals exhibited significant glomerulosclerosis, tubulo-interstitial injury and heavy mononuclear cell infiltration.

**Figure 2** - Bar graphs depicting plasma malondialdehyde (MDA) concentration and reduced/oxidized glutathione ratio as well as thiobarbituric acid reactive substances (TBARS) in the whole renal cortical tissue homogenate and in the isolated mitochondria from renal cortex in the CRF and control groups at 12 weeks post renal ablation or sham operation. 

n = 6 in each group. Data are presented as mean ± SD. *p<0.05, **p<0.01.

**Figure 3** – Representative Western blots and group data depicting protein abundance of the NAD(P)H oxidase subunits (NOX-4, gp91phox, p22phox, p47phox, and rac1) in the renal tissues of the 5/6 nephrectomized (CRF) and control (CTL) rats at 12 weeks post renal ablation.

n = 6 in each group. Data are presented as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

**Figure 4** – Representative Western blots and group data depicting protein abundance of MCP-1, COX-2, and 12 lipoxygenase in the renal tissues of the 5/6 nephrectomized (CRF) and control (CTL) rats at 12 weeks post renal ablation.

n = 6 in each group. Data are presented as mean ± SD. **p<0.01, ***p<0.001, ***p<0.001.
Figure 5 – Representative Western blots and group data depicting protein abundance of phospho-IκB and nuclear contents of p65 active subunit of NFκB in the renal tissues of the 5/6 nephrectomized (CRF) and control (CTL) rats at 6 and 12 weeks post renal ablation. 

n = 6 in each group. Data are presented as mean ± SD. *p<0.05, **p<0.01.

Figure 6 – Representative Western blots and group data depicting protein abundance of gp91phox, p47phox, Rac-1, MCP-1, COX-2 and PAI-1 in the renal tissues of the 5/6 nephrectomized (CRF) and control (CTL) rats at 6 weeks post renal ablation.

n = 6 in each group. Data are presented as mean ± SD. *p<0.05.

Figure 7 – Representative Western blots and group data depicting nuclear translocation of Nrf2 and protein abundance of its repressor, Keap1, in the renal tissues of the CRF and CTL rats at 6 weeks (upper panel) and 12 weeks (lower panel).

n = 6 in each group. *p<0.01, ***p<0.001

Figure 8 – Representative Western blots and group data depicting protein abundance of heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase (NQO1)and catalytic (GCLC) and modulatory (GCLM) subunits of glutamate-cysteine ligase in the renal tissues of the CRF and CTL rats at 12 weeks.

n = 6 in each group. *p<0.05, **p<0.01

Figure 9 – Representative Western blots and group data depicting protein abundance of Cu,ZnSOD, Mn-SOD, extracellular SOD, catalase and glutathione peroxidase in the renal tissues of the 5/6 nephrectomized (CRF) and control (CTL) rats at 12 weeks.
n = 6 in each group. *p<0.05, **p<0.01.

**Figure 10** - Representative Western blots and group data depicting protein abundance of catalytic (GCLC) and modulatory (GCLM) subunits of glutamate-cysteine ligase, Mn-SOD, Catalase, Cu,Zn-SOD, and EC-SOD in the renal tissues of the 5/6 nephrectomized (CRF) and control (CTL) rats at 6 weeks.

n = 6 in each group. *p<0.05

**Figure 11** - Schematic representation of Nrf2 and NF-κB activation by reactive oxygen species (ROS), electrophiles and endoplasmic reticulum stress (ER stress). Under normal condition, Nrf2 is sequestered in the cytoplasm via binding to its repressor molecule, Keap1. Oxidative, electrophilic and ER stress (as well as Nrf2 phosphorylation by protein kinases) cause dissociation of Nrf2-Keap1 complex which culminates in ubiquitination of Keap1 and nuclear translocation of Nrf2. After heterodimerization with other transcription factors such as small Maf, within the nucleus, Nrf2 promotes transcriptional activation of antioxidant and detoxifying enzymes by binding to the antioxidant responsive elements (ARE) in the promoter regions of the target genes. Simultaneously via phosphorylation of the repressor molecule, I-κB, oxidative and electrophilic stress causes activation of NF-κB leading to transcriptional activation of genes encoding inflammatory cytokine and chemokines. There is evidence that the Nrf2 and NF-κB pathways exert mutual inhibitory influence on one another.
Table 1- Serum concentrations of creatinine (Cr), urea, albumin, triglyceride, cholesterol and creatinine clearance (Ccr), urine protein, systolic (S), diastolic (D) blood pressure (BP), weight gain and kidney weight at 6 weeks (upper panel) and 12 weeks (lower panel). Values are given as mean ± S.E. (n=5-7 in each group).

<table>
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<th>GROUPS (at 6 weeks)</th>
<th>CTL</th>
<th>CRF</th>
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<tr>
<td>Cr (mg/dL)</td>
<td>0.93 ± 0.07</td>
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<td>Urea (mg/dL)</td>
<td>69.2 ± 3.9</td>
<td>127.1 ± 5.1***</td>
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<td>Ccr (ml/min/kg)</td>
<td>2.19 ± 0.35</td>
<td>1.31 ± 0.11*</td>
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<td>Albumin (g/dL)</td>
<td>2.78 ± 0.10</td>
<td>2.66 ± 0.07</td>
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<td>Urine protein (mg/24h)</td>
<td>37.0 ± 6.2</td>
<td>100.7 ± 9.0*</td>
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<td>Triglycerides (mg/dL)</td>
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<td>41.1 ± 3.7</td>
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<td>Total cholesterol (mg/dL)</td>
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<td>SBP (mmHg)</td>
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<td>DBP (mmHg)</td>
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<td>Weight gain (g)</td>
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<td>Kidney weight (g)</td>
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<td>1.22 ± 0.04b</td>
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<th>GROUPS (at 12 weeks)</th>
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<td>Cr (mg/dL)</td>
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<tr>
<td>Urea (mg/dL)</td>
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<td>Ccr (ml/min/kg)</td>
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<td>Triglycerides (mg/dL)</td>
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<td>221.2 ± 10.3***</td>
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<td>Weight gain (g)</td>
<td>146.0 ± 6.0</td>
<td>119.3 ± 6.0*</td>
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<tr>
<td>Kidney weight (g)</td>
<td>1.45 ± 0.07a</td>
<td>2.38 ± 0.19***</td>
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*p<0.05; **p<0.01; ***p<0.001 vs CTL groups. aIntact kidney weight in the CTL groups. bRemnant kidney weight in the CRF groups.
Figure 1
Figure 2

Kidney tissue TBARS (nmol/mg protein)

Plasma MDA (nmol/mL)

Mitochondrial TBARS (nmol/mg protein)

Reduced GSH/GSSG ratio
Figure 3
Figure 4
Figure 5

(A) 6 weeks

- **p-IκB**
  - CTL
  - CRF

- **NF-κB p65**
  - CTL
  - CRF

- **GAPDH**
  - CTL
  - CRF

- **Histone H1**
  - CTL
  - CRF

(B) 12 weeks

- **p-IκB**
  - CTL
  - CRF

- **NF-κB p65**
  - CTL
  - CRF

- **β-actin**
  - CTL
  - CRF

- **Histone H1**
  - CTL
  - CRF

**Relative optical density**

- **CTL**
- **CRF**

* and ** indicate statistical significance.
Figure 6

The figure shows a comparison of relative optical density for various proteins under different conditions. The proteins include gp91phox, p47phox, Rac-1, GAPDH, MCP-1, COX2, PAI-1, and GAPDH. The graph indicates differences between control (CTL) and CRF-treated samples.
Figure 7

(A) 6 weeks

(B) 12 weeks
Figure 8

NQO1

HO-1

β-actin

NQO1

β-actin

Relative optical density

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

CTL CRF

Relative optical density

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

CTL CRF

GCLC

β-actin

GCLM

β-actin

Relative optical density

0.0 0.2 0.4 0.6 0.8 1.0 1.2

CTL CRF

Relative optical density

0.0 0.2 0.4 0.6 0.8 1.0 1.2

CTL CRF

* p < 0.05

** p < 0.01
Figure 9

Cu,Zn-SOD

Mn-SOD

EC-SOD

Catalase

Gpx

Relative optical density

CTL CRF

Relative optical density

CTL CRF

Relative optical density

CTL CRF

Figure 9
Figure 10

The image shows a bar chart with the following data points:

- GCLC
- GCLM
- GAPDH
- Cu,zn-SOD
- Mn-SOD
- EC-SOD
- Catalase
- GAPDH

The chart compares two conditions: CTL and CRF, with the y-axis representing relative optical density.
Figure 11