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

Submitted 24 July 2009; accepted in final form 5 December 2009

Kim HJ, Vaziri ND. Contribution of impaired Nrf2-Keap1 pathway to oxidative stress and inflammation in chronic renal failure. Am J Physiol Renal Physiol 298: F662–F671, 2010. First published December 9, 2009; doi:10.1152/ajprenal.00421.2009.—Oxidative stress and inflammation are constant features and major mediators of progression of chronic kidney disease (CKD). Nuclear factor erythroid-2-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 wk. 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, NF-κB activation, mononuclear cell infiltration, and one depletion, NF-κB stress were studied. In confirmation of earlier studies, rats with

Oxidative or covalent modification of thiols in some of these enzymes 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 (HO-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 intracellular redox state. Oxidative or covariant 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 or electrophile response elements, 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). Although pathways involved in intrarenal 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.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats, weighing 225–250 g, were purchased from Harlan Sprague Dawley (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
Table 1. Serum concentrations of creatinine, urea, albumin, triglyceride, cholesterol and creatinine clearance, urine protein, systolic and diastolic blood pressure, weight gain, and kidney weight at 6 and 12 wk

<table>
<thead>
<tr>
<th>Groups at 6 wk</th>
<th>CTL</th>
<th>CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr, mg/dl</td>
<td>0.93 ± 0.07</td>
<td>1.40 ± 0.02*</td>
</tr>
<tr>
<td>Urea, mg/dl</td>
<td>69.2 ± 3.9</td>
<td>127.1 ± 5.1#</td>
</tr>
<tr>
<td>Ccr, ml/min kg⁻¹</td>
<td>2.19 ± 0.35</td>
<td>1.31 ± 0.11*</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>2.78 ± 0.10</td>
<td>2.66 ± 0.07</td>
</tr>
<tr>
<td>Urine protein, mg/24 h</td>
<td>37.0 ± 6.2</td>
<td>100.7 ± 9.0*</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>33.8 ± 5.1</td>
<td>41.1 ± 3.7</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>87.8 ± 7.1</td>
<td>111.1 ± 5.5*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>126.7 ± 6.2</td>
<td>150.3 ± 5.5*</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>103.8 ± 10.0</td>
<td>128.6 ± 5.8</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>162.3 ± 5.1</td>
<td>147.9 ± 4.7</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.14 ± 0.02</td>
<td>1.22 ± 0.04</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups at 12 wk</th>
<th>CTL</th>
<th>CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr, mg/dl</td>
<td>0.50 ± 0.06</td>
<td>1.56 ± 0.23‡</td>
</tr>
<tr>
<td>Urea, mg/dl</td>
<td>54.3 ± 1.8</td>
<td>114.7 ± 4.8#</td>
</tr>
<tr>
<td>Ccr, ml/min kg⁻¹</td>
<td>5.62 ± 0.53</td>
<td>1.74 ± 0.19‡</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>2.76 ± 0.09</td>
<td>2.34 ± 0.11†</td>
</tr>
<tr>
<td>Urine protein, mg/24 h</td>
<td>6.7 ± 0.9</td>
<td>80.3 ± 3.7‡</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>45.8 ± 8.2</td>
<td>99.7 ± 2.1*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>712.4 ± 6.0</td>
<td>221.2 ± 10.3‡</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>123.5 ± 6.0</td>
<td>161.4 ± 2.0†</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>87.5 ± 3.3</td>
<td>117.0 ± 3.2‡</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>146.0 ± 6.0</td>
<td>119.3 ± 6.0‡</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.45 ± 0.07</td>
<td>2.38 ± 0.19†</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 5–7 in each group). Cr, creatinine; Ccr, creatinine clearance; SBP and DBP, systolic and diastolic blood pressure. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. control (CTL) groups. Intact kidney weight was measured in CTL groups, while remnant kidney weight was measured in the chronic renal failure (CRF) groups.
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, monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), Nrf2, Keap1, HO-1, NQO1, GCLC, and GCLM antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against 12-lipoxygenase (12-LO; Cayman Chemical, Ann Arbor, MI), Cu,Zn-SOD and catalase.

Fig. 2. Bar graphs depicting plasma malondialdehyde (MDA) concentration and reduced (GSH)-to-oxidized glutathione (GSSG) 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 wk postrenal ablation or sham operation. Data are means ± SD; n = 6 in each group. *P < 0.05, **P < 0.01.

Fig. 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 and control rats at 12 wk postrenal ablation. Data are means ± SD; n = 6 in each group. *P < 0.05, **P < 0.01, ***P < 0.001.
(EMD Chemicals, Gibbstown, NJ), glutathione peroxidase (Gpx) and Mn-SOD (Millipore, Billerica, MA), phospho-IκB-α (Cell Signaling Technology, Denver, CO), and gp91phox, p47phox, and Rac1 (BD Bioscience, San Jose, CA) were purchased from the cited sources. Antibodies to histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Sigma, St. Louis, MO), 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, San Diego, CA) at 120 V for 2 h and transferred to a Hybond-ECL membrane (Amersham Life Science, Arlington Heights, IL). The membrane was incubated for 1 h in blocking buffer (1 × 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 min in 1 × TBS, 0.05% Tween-20 before a 2-h incubation in a buffer (1 × TBS, 0.05% Tween-20 and 3% nonfat milk) containing horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG (Amersham Life Science) at 1:1,000 dilution. The membrane was washed four times and developed by autoradiography using the ECL chemiluminescent agents (Amersham Life Science).

Data analysis. A Student’s t-test was used in statistical evaluation of the data, which are shown as means ± SD, unless otherwise specified. P values <0.05 were considered significant.

Fig. 4. Representative Western blots and group data depicting protein abundance of monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), and 12-lipoxygenase (12-LPO) in the renal tissues of the 5/6 nephrectomized and control rats at 12 wk postrenal ablation. Data are means ± SD; n = 6 in each group. **P < 0.01, ***P < 0.001.

Fig. 5. Representative Western blots and group data depicting protein abundance of phospho-IκB and nuclear contents of p65 active subunit of NF-κB in the renal tissues of the 5/6 nephrectomized and control rats at 6 (A) and 12 (B) wk postrenal ablation. Data are means ± SD; n = 6 in each group. *P < 0.05, **P < 0.01.
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 (Fig. 1).

Markers of oxidative stress. Data are shown in Fig. 2. Compared with 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 with those found in the control group. In confirmation of our earlier studies (42), the plasma concentration of nitrotyrosine, a footprint of nitric oxide interaction with ROS, 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 (GSSG) was increased and the GSH-to-GSSG 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 shown in Figs. 3, 4, and 5. Compared with the control group, the CRF group studied 12 wk 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 proinflammatory and profibrotic cytokine and other mediators. Examination of renal tissues obtained at 6 wk after renal ablation revealed directionally similar but less intense changes in the above parameters (Figs. 5 and 6).

Nrf2/Keap1 pathway. Data are shown in Figs. 7, 8, 9, and 10. Compared with 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 wk. The CRF-induced reduction of nuclear Nrf2 abundance and elevation of cytoplasmic Keap1 abundance were more intense at 12 wk. This was associated with significant reduction of renal tissue HO-1, GCLC, GCLM, NQO1, catalase, GPX, Cu,Zn-SOD, and Mn-SOD protein abundance in the remnant kidney at 12 wk post-5/6 nephrectomy. Changes in the above parameters in the remnant kidney tissues at 6 wk following renal ablation were less intense (Figs. 5 and 6). Conspicuous failure of the Nrf2 activation at 6 wk postrenal ablation and marked reduction of its activity at 12 wk 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 that 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 and NQO1), and antioxidant enzymes (CAT, GPx, SOD, and HO-1). Via activation of Nrf2 and consequent expression of the antioxidant and detoxifying enzymes, ROS 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 (Fig. 11). For instance, the inflammatory response to activation of NF-κB and consequent induction of COX-2, inducible nitric oxide synthase, IL-6, and TNF-α is more intense in Nrf2 knockout 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 wk after 5/6 nephrectomy exhibited oxidative stress, inflammation, NF-κB activation, and upregulation of COX-2, 12-LO, and the ROS-generating enzyme...
Fig. 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 wk; n = 6 in each group. *P < 0.05, **P < 0.01.

Fig. 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 wk (A) and 12 wk (B); n = 6 in each group. *P < 0.01, ***P < 0.001.
NAD(P)H oxidase in the remnant kidney. While pathways involved in intrarenal 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 and was explored here. Despite severe oxidative stress and inflammation which should have induced activation of Nrf2 and upregulation of its downstream 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 downregulation of the Nrf2 target gene products including the antioxidant enzymes (catalase, SOD, GPX, and HO-1), glutamate-cysteine ligase (GCLC and GCLM), the key enzyme in glutathione synthesis, and the detoxifying enzyme NQO1 at 12 wk and a less severe decline or lack of expected rise at 6 wk. 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 a significant elevation of Keap1 abundance in the remnant kidneys of the CRF animals. Keap1 is a cysteine-rich protein that serves as a redox sensor, an inhibitor of nuclear translocation of Nrf2 and a facilitator of its proteasomal degradation (13). Therefore, the 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 knockout mice (46,
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 the 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 the enzymes of the glutathione redox cycle heightens the susceptibility to ROS-mediated cell injury (8), and many pathological 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-to-GSSG ratio (reflecting depressed cellular redox status) in the renal tissue.

In addition to regulating the 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 the 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 downregulation 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, the presence of uncontained oxidized lipids and lipoproteins in the remnant kidney can lead to irrepressible lipid uptake by macrophages and resident cells via upregulation 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 upregulation of MCP-1, COX-2,
and 12-LO representing intense inflammatory reaction. ROS, particularly hydroperoxides, are potent activators of the redox-sensitive transcription factor NF-κB, the general transcription factor for many proinflammatory cytokines and chemokines. Thus the 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 that 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 proinflammatory 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 downstream antioxidant molecules. Accordingly, impaired Nrf2 activation shown for the first time here contributes to the severity of oxidative stress and inflammation and the 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.

DISCLOSURES
No conflicts of interest are declared by the authors.

REFERENCES
34. Sindhu RK, Ehladea A, Harmand F, Dhaliwal KK, Nguyen T, Zhan CD, Roberts CK, Vaziri ND. Expression of catalase and glutathione


