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Authors
Aminzadeh, MA
Nicholas, SB
Norris, KC
et al.

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Role of impaired Nrf2 activation in the pathogenesis of oxidative stress and inflammation in chronic tubulo-interstitial nephropathy

Mohammad A. Aminzadeh\textsuperscript{1},
Susanne B. Nicholas\textsuperscript{2,3},
Keith C. Norris\textsuperscript{2,3}
and Nosratola D. Vaziri\textsuperscript{1}

\textsuperscript{1}Division of Nephrology and Hypertension, University of California, Irvine, CA, USA,
\textsuperscript{2}Department of Medicine, University of California, Los Angeles, CA, USA and
\textsuperscript{3}Charles R. Drew University of Medicine and Science, Los Angeles, CA, USA

Correspondence and offprint requests to: Nosratola D. Vaziri; E-mail: ndvaziri@uci.edu

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ABSTRACT

**Background.** Tubulo-interstitial nephropathy (TIN) is a common cause of chronic kidney disease (CKD). Consumption of an adenine-containing diet causes the accumulation of 2,8-dihydroxyadenine in the renal tubules triggering intense chronic TIN and progressive CKD in rats. CKD in this model is associated with, and largely driven by, oxidative stress and inflammation. Oxidative stress and inflammation in rats with spontaneous focal segmental glomerulosclerosis and rats with CKD induced by 5/6 nephrectomy are associated with an impaired activation of nuclear factor-erythroid-2-related factor 2 (Nrf2) which is the master regulator of genes encoding many antioxidant and detoxifying enzymes. The effect of TIN on the Nrf2 pathway and its key target genes is unknown and was investigated here.

**Methods.** Sprague-Dawley rats were randomized to control and adenine-treated (rat chow-containing 0.7% adenine for 2 weeks) groups and followed up for 4 weeks.

**Results.** The adenine-treated animals exhibited marked azotemia, impaired urinary concentrating capacity, intense tubular and glomerular damage, interstitial inflammation and fibrosis. This was associated with an increased expression of NAD(P)H oxidase, cyclooxygenase-2 and 12-lipoxygenase, and activation of NF-\(\kappa\)B, the master regulator of pro-inflammatory cytokines and chemokines. Oxidative stress and inflammation in the kidneys of adenine-treated animals was accompanied by an impaired activation of Nrf2 and down-regulation of its target gene products including, catalase, heme oxygenase-1 and glutamate-cysteine ligase.

**Conclusions.** Chronic TIN is associated with impaired Nrf2 activity which contributes to the pathogenesis of oxidative stress and inflammation and amplifies their damaging effects on the kidney.

INTRODUCTION

Oxidative stress and inflammation play a central part in the pathogenesis and progression of chronic kidney disease (CKD) [1–4]. Under physiological conditions, oxidative stress triggers up-regulation of the endogenous antioxidant and cytoprotective proteins and enzymes to prevent or limit tissue injury and dysfunction. This process is mediated by activation of the nuclear factor-erythroid-2-related factor 2 (Nrf2) which regulates the basal activity and coordinated induction of numerous genes that encode various antioxidant and phase 2 detoxifying enzymes and related proteins [5, 6]. Nrf2 is held as an inactive complex in the cytoplasm by the repressor molecule, Keap1 (Kelch-like ECH-associated protein 1) which facilitates its ubiquitination. Keap1 contains a number of reactive cysteine residues which function as intracellular redox sensors. Oxidative or covalent modification of thiols in some of these cysteine residues results in disruption of one of the two Keap1 interaction sites with Nrf2 (hinge and latch model), thereby limiting binding and proteosomal degradation.
of Nrf2. This leads to accumulation of free Nrf2 and its translocation to the nucleus [7–9]. In the nucleus, Nrf2 heterodimerizes with other transcription factors (e.g. small Maf) and binds to the regulatory sequences known as antioxidant response elements (AREs) or electrophile response elements, in the promoter regions of its target genes. In addition to modification of Keap1, nuclear translocation of Nrf2 may occur via phosphorylation of its threonine or serine residues by upstream kinases, such as protein kinase C, mitogen-activated protein kinases, phosphatidylinositol-3-kinase/Akt, casein kinase-2 and the endoplasmic reticulum enzyme, PKR-like ER kinase [10, 11]. Regulation of cellular antioxidant and anti-inflammatory machinery by Nrf2 plays a central part in defense against oxidative stress [5]. In fact, Nrf2 disruption in mice attenuates or abrogates the induction of genes encoding antioxidants in response to oxidative stress. In addition, ablation of the Nrf2 gene causes a lupus-like autoimmune nephritis and exacerbates diabetes-induced oxidative stress, inflammation and nephropathy in experimental animals [12, 13].

Progression of renal disease in Imai rats with spontaneous focal segmental glomerulosclerosis [14] and in rats with CKD induced by 5/6 nephrectomy [15–17] is associated with severe oxidative stress and inflammation in the kidney tissue. However, despite severe oxidative stress which should lead to Nrf2 activation, Nrf2 activity and expression of its target gene products are reduced in the diseased kidney tissues in these models [18–20].

Tubulo-interstitial nephropathy (TIN) is a relatively common cause of CKD in humans. Rats or mice fed an adenine-containing diet exhibit severe TIN resembling that seen in humans [21–25]. The TIN in this model is mediated by the renal tubular precipitation of dihydroxyadenine leading to tubular epithelial cell injury, interstitial inflammatory cell infiltration, fibrosis and progressive deterioration of kidney function [26, 27]. As with many other forms of CKD, progression of renal disease in this model is largely driven by inflammation and oxidative stress. Oxidative stress and inflammation are inseparably linked as they form a vicious cycle in which oxidative stress provokes inflammation by several mechanisms including activation of the transcription factor kappa B (NF-κB) which leads to the activation and recruitment of immune cells. Inflammation, in turn, provokes oxidative stress via production of reactive oxygen, nitrogen and halogen species by the activated leukocytes and resident cells. Together these events promote tissue damage by inflicting apoptosis, necrosis and fibrosis.

Clinical and histopathological features of CKD caused by chronic TIN are distinctly different from those caused by other disorders. To our knowledge the Nrf2 pathway in chronic TIN has not been previously studied. The present investigation was conducted to assess the effect of chronic TIN on the Nrf2-Keap1 pathway.

### Materials and Methods

#### Animals

Male Sprague-Dawley rats, weighing 225–250 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA). They were housed in a climate-controlled and light-regulated facility with 12:12-h day–night cycles. The animals were randomized to the CKD and control groups. The animals assigned to the CKD group were fed rat chow containing 0.7% adenine for 2 weeks and followed for an additional 2 weeks on a regular diet. The control animals were fed regular rat chow throughout the observation period. Six animals were included in each group. At the conclusion of the observation period, animals were placed in metabolic cages for a 24-h urine collection. They were then anesthetized (Ketamine/Xylazine IP) and euthanized by exsanguinations using cardiac puncture. The kidneys were immediately removed and processed for histological evaluation and western blot analyses. All experiments were approved by the University of California Irvine Institutional Committee for

| Table 1. Plasma concentrations of creatinine and urea, creatinine clearance, urine volume, urinary protein excretion, systolic arterial pressure, hematocrit and body weight in the control (CTL) rats and rats with adenine-induced chronic interstitial nephropathy (TIN) |
|-----------------------------------------------|-------------------|-------------------|
|                                | CTL (n = 6) | TIN (n = 6) |
| Plasma creatinine (mg/dL)            | 0.29 (0.08) | 1.25 (0.33)† |
| Plasma urea (mg/dL)                  | 49.95 (23.71) | 80.56 (40.21)† |
| Creatinine clearance (mL/min)        | 2.50 (1.00) | 0.50 (0.36)† |
| Urine volume (mL)                    | 7.95 (2.45) | 38.60 (17.11)† |
| Protein/creatinine ratio (g/mg)      | 6.10 (1.25) | 8.45 (1.87)† |
| Systolic blood pressure (mmHg)       | 127.00 (16.50) | 146.00 (14.50)† |
| Hematocrit (%)                       | 45 (3.60) | 32 (3.60) |
| Body weight (g)                      | 460 (25) | 417.50 (22.25)† |

Values are median (inter-quartile range).

*P < 0.01 versus CTL.
the Use and Care of Experimental Animals. Plasma glutathione (GSH) and oxidized glutathione (GSSG) levels were measured by high-performance liquid chromatography and serum creatinine, urea and urinary protein excretion were measured as described previously [28]. Systolic blood pressure was determined by tail plethysmography at 2 and 4 weeks as described previously [29].

Western blot analyses

Cytoplasmic and nuclear extracts were prepared as described previously [30]. 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, NOX4, Keap1, catalytic subunit of glutamate-cysteine ligase (GCLC), cyclooxygenase-2 (COX-2) and heme oxygenase-1 (HO-1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phosphorylated IκB-α (p-IκB-α; Cell Signaling Technology, Denver, CO, USA), 12-lipoxygenase (12-LO; Cayman Chemical, Ann Arbor, MI, USA), catalase (EMD Chemicals, Gibbstown, NJ, USA), gp91phox, p67phox and Rac1 (BD Bioscience, San Jose, CA, USA) were purchased from the cited sources. Antibodies to histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 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, USA) at 120 V for 2 h and transferred to a Hybond-ECL membrane (Amersham Life Science, Arlington Heights, IL, USA). The membrane was incubated for 1 h in blocking buffer (1× TBS, 0.05% Tween-20 and 5% non-fat 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% non-fat milk) containing horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG (Amersham Life Science) at 1:1000 dilution. The membrane was washed four times and developed by autoradiography using the ECL chemiluminescent agents (Amersham Life Science).

Statistical analysis

The Mann–Whitney U test was used in statistical analysis of the data which are presented as Box-plot diagrams and median followed by inter-quartile range. P-values < 0.05 were considered significant. Box-plot diagrams were employed in presentation of the graphical data. Each box encompasses the range of values from the 25% percentile (lower bar) to the 75% percentile (upper bar), the horizontal line represents the median value and the lines above and below the box signify the maximum and minimum values.

RESULTS

General data

Compared with the control group, the rats with adenine-induced TIN exhibited significantly higher plasma urea and

FIGURE 2: Representative photomicrographs of periodic acid schiff (PAS)-stained kidney sections from a rat with adenine-induced TIN (TIN) and a control (CTL) rat. The kidney in the TIN animals exhibited significant tubulo-interstitial injury marked by tubular dilation, heavy inflammatory cell infiltration and fibrosis. Magnification ×200.
creatinine concentrations, urine volume, and arterial pressure and a significantly lower hematocrit and body weight (Table 1). This was associated with a significant elevation of the plasma oxidized glutathione level and oxidized to the reduced glutathione ratio (Figure 1). As expected, the histological examination of the kidney tissue revealed severe tubulo-interstitial injury, marked tubular dilation and heavy inflammatory cell infiltration in the adenine-treated group (Figure 2).

Oxidative and inflammatory pathways

Data are shown in Figures 3 and 4. Compared with the control group, the rats with adenine-induced TIN showed a significant increase in phosphorylated IkB and nuclear p65 contents denoting NF-κB activation in the kidney tissue. This was accompanied by a significant increase in protein abundance of the NAD(P)H oxidase subunits (NOX4, gp91phox, p67phox and Rac1) and significant up-regulation of 12-LO, and COX-2 in the kidneys of rats with adenine-induced TIN compared with those found in the control group.

Nrf2/Keap1 pathway

Data are shown in Figure 5. Compared with the control animals, the rats with adenine-induced TIN exhibited a significant reduction in the nuclear Nrf2 content and a marked increase in the Keap1 abundance in the kidney tissue. This was associated with significant down-regulation of the measured Nrf2 target gene products including catalase, HO-1 and GCLC in the kidneys of rats with adenine-induced chronic TIN. These findings point to the impaired activation of the Nrf2 pathway in this model.

**Figure 3:** Representative western blots and group data depicting protein abundance of the NAD(P)H oxidase subunits (NOX-4, Rac1, p67phox and gp91phox) in the renal tissues of rats with adenine-induced TIN (TIN) and control (CTL) rats n = 6 in each group. **P < 0.01.
DISCUSSION

The adenine-treated animals showed marked azotemia, polyuria, anemia, hypertension and minimal proteinuria. The histological examination revealed severe tubulo-interstitial injury marked by tubular dilation, massive inflammatory cell infiltration and fibrosis in the renal tissue. This was associated with the activation of NF-κB as evidenced by increased phosphorylation of its repressor molecule, IκB, and nuclear translocation of p65 active subunit along with up-regulation of COX-2, 12-lipoxygenase and NAD(P)H oxidase, in the renal tissue.

Intense up-regulation of oxidative and inflammatory pathways in the kidney of rats with adenine-induced TIN was compounded by severe impairment of the Nrf2 activity as evidenced by marked reduction in its nuclear content and elevation of its suppressor molecule, Keap1. As expected, impaired Nrf2 activity in the kidneys of rats with adenine-induced chronic TIN was associated with down-regulation of its measured target gene products including catalase, heme oxygenase-1 and glutamate-cysteine ligase. These enzymes protect against oxidative stress-induced cytotoxicity and tissue injury either directly by neutralizing reactive oxygen species or indirectly by catalyzing production of antioxidant and detoxifying substrates. For instance, catalase directly converts hydrogen peroxide to water and thus prevents its conversion to highly cytotoxic products such as hydroxyl radical in the presence of transition metals or to hypochlorous acid in the presence of myeloperoxidase which is abundant in macrophages and polymorphonuclear leukocytes. On the other hand, heme oxygenase-1 and glutamate-cysteine ligase confer protection by, respectively, catalyzing the formation of bilirubin and glutathione which are potent antioxidant and detoxifying agents. Thus, the observed impairment of the

FIGURE 4: Representative western blots and group data depicting protein abundance of cyclooxygenase-2 (COX-2), 12-lipoxygenase (12-LO), phospho-IκB and nuclear content of p65 active subunit of NF-κB in the renal tissues of rats with adenine-induced TIN (TIN) and control (CTL) rats n = 6 in each group. **P < 0.01.
Nrf2 activity and consequent down-regulation of its antioxidant and detoxifying target gene products play a major role in the pathogenesis and progression of kidney disease in this model. The conspicuous impairment of Nrf2 activity in the face of the prevailing oxidative stress and inflammation in this model of chronic TIN recapitulates our previous findings in the Imai rats with spontaneous progressive focal segmental glomerulosclerosis and rats with CKD induced by 5/6 nephrectomy [18, 19]. The presence of impaired Nrf2 activity as a shared feature in distinctly different forms of CKD points to its role as a common mediator of progression of kidney disease of diverse etiologies. If true, strategies aimed at restoring Nrf2 activity can be effective in preventing/retarding CKD progression [31]. The renal protective effect of Nrf2 is supported by the fact that Nrf2 gene ablation intensifies the diabetes-induced inflammation, oxidative stress and renal injury in the experimental animals [12], that the Nrf2 knockout mice exhibit a lupus-like autoimmune nephritis [13], and that 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 [32].

Besides increasing the vulnerability of the diseased kidney to the direct ravages of oxidative stress, impaired Nrf2 activation contributes to the development and amplification of intra-renal inflammation by promoting accumulation of hydroperoxides and lipoperoxides which are potent activators of NF-κB. In fact, several studies have demonstrated the anti-inflammatory function of Nrf2 [33–35]. The precise mechanism responsible for the impaired renal tissue Nrf2 activity in the kidneys of our animals with chronic TIN is presently unclear. It should be noted that impaired Nrf2 activity has been demonstrated in several chronic inflammatory disorders such as chronic granulomatous disease [36] and asthma [37]. This is due to the interference of the NF-κB p65 and p53 subunits with the dissociation of Nrf2 from Keap 1 and binding of Nrf2 to the AREs of the target genes [38, 39]. Thus, the failure of the physiological activation of Nrf2 despite the prevailing oxidative stress in the diseased kidney appears to be at least, in part, due to the accompanying systemic inflammation.

In conclusion, chronic TIN is associated with impaired Nrf2 activity that contributes to the pathogenesis of oxidative stress and inflammation and amplifies their damaging effects on the kidney.

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**FIGURE 5**: Representative western blots and group data depicting nuclear translocation of Nrf2 and protein abundances of its repressor, Keap1, and its downstream gene products, catalase, heme oxygenase-1 (HO-1) and catalytic (GCLC) subunit of glutamate-cysteine ligase in the renal tissues of the adenine-treated (TIN) and control (CTL) rats; n = 6 in each group. **P < 0.01.
CONFLICT OF INTEREST STATEMENT

None declared.

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