Role of Intrarenal Angiotensin System Activation, Oxidative Stress, Inflammation, and Impaired Nuclear Factor-Erythroid-2-Related Factor 2 Activity in the Progression of Focal Glomerulosclerosis

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ABSTRACT

The Imai rat is a model of spontaneous focal glomerulosclerosis, which leads to heavy proteinuria, hyperlipidemia, hypertension, and progressive renal failure. Treatment with AT1 blockers (ARBs) ameliorates proteinuria, hyperlipidemia, and nephropathy in this model. Progression of renal disease in 5/6 nephrectomized rats is associated with activation of the intrarenal angiotensin system, up-regulation of the oxidative, inflammatory, and fibrogenic pathways, and impaired activity of nuclear factor-erythroid-2-related factor 2 (Nrf2), the master regulator of genes encoding antioxidant molecules. We hypothesized that progressive nephropathy in the Imai rat is accompanied by oxidative stress, inflammation, and impaired Nrf2 activation and that amelioration of nephropathy with AT1 receptor blockade in this model may be associated with the reversal of these abnormalities. Ten-week-old Imai rats were randomized to the ARB-treated (olmesartan, 10 mg/kg/day for 24 weeks) or vehicle-treated groups. Sprague-Dawley rats served as controls. At 34 weeks of age Imai rats showed heavy proteinuria, hypoalbuminemia, hypertension, azotemia, glomerulosclerosis, tubulointerstitial inflammation, increased angiotensin II expressing cell population, up-regulations of AT1 receptor, AT2 receptor, NAD(P)H oxidase, and inflammatory mediators, activation of nuclear factor-κB and reduction of Nrf2 activity and expression of its downstream gene products in the renal cortex. ARB therapy prevented nephropathy, suppressed oxidative stress and inflammation, and restored Nrf2 activation and expression of the antioxidant enzymes. Thus progressive focal glomerulosclerosis in the Imai rats is associated with oxidative stress, inflammation, and impaired Nrf2 activation. These abnormalities are accompanied by activation of intrarenal angiotensin system and can be prevented by ARB administration.

Introduction

The Imai rat is a model of spontaneous focal glomerulosclerosis, which leads to nephrotic syndrome, severe hyperlipidemia, hypertension, and progressive renal failure. These animals develop proteinuria and hyperlipidemia and progressive focal segmental glomerulosclerosis at 6 to 8 weeks of age, culminating in end-stage renal disease and death by 8 to 9 months of age (Yoshikawa and Yamasaki, 1991). In an earlier study we found marked amelioration of proteinuria, hyperlipidemia, and nephropathy with long-term AT1 receptor blockade in this model (Rodríguez-Iturbe et al., 2004). Blockade of the rennin-angiotensin system is highly effective in retarding progression of renal disease in humans and experimental animals (Remuzzi et al., 2005; Wolf and Ritz, 2005). The salutary effect of rennin-angiotensin system blockade is not only caused by its antihypertensive/hemodynamic actions, but also by its ability to reduce oxidative stress, inflammation, and fibrosis, which are major mediators of progression of renal disease (Esteban et al., 2003; Remuzzi et al., 2005; Hayashi et al., 2010). Several studies have demonstrated activation of the intrarenal angiotensin system marked by concomitant up-regulation of angiotensin

ABBREVIATIONS: ROS, reactive oxygen species; ARB, AT1 blocker; Nrf2, nuclear factor-erythroid-2-related factor 2; NF-κB, nuclear factor-κB; SOD, superoxide dismutase; Keap1, Kelch-like erythroid cell-derived-associated protein 1; TBARS, thiobarbituric acid reactive substance; MCP-1, monocyte chemoattractant protein-1; COX-2, cyclooxygenase-2; HO-1, heme oxygenase-1; 12-LO, 12-lipoxygenase; GCLC, glutamate-cysteine ligase catalytic subunit; GCM, glutamate-cysteine ligase modifier subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBA, thiobarbituric acid; TBS, Tris-buffered saline; CTL, control.
II and AT1 receptor expression along with activation/up-regulation of oxidative, inflammatory, and fibrogenic pathways in the diseased kidney and their amelioration with angiotensin blockade (Gonçalves et al., 2004; Xu et al., 2005; Vaziri et al., 2007; Yu et al., 2007).

Binding of angiotensin II to AT1 receptor increases the production of reactive oxygen species (ROS) via protein kinase C-mediated activation of NAD(P)H oxidase that colocalizes with the AT1 receptor in the cell membrane (Garriodo and Griendling, 2009). Excessive production of ROS causes tissue damage and dysfunction and promotes necrosis, apoptosis, inflammation, and fibrosis by activating redox-sensitive transcription factors and signal transduction pathways and by denaturing and modifying the structural and functional molecules. For instance ROS initiate inflammation by activating NF-κB via phosphorylation of NF-κB inhibitor (Pantano et al., 2006).

Under normal condition the organism is protected against ROS-induced tissue injury by the natural antioxidant defense system, which consists of numerous antioxidant enzymes, phase II detoxifying agents, and antioxidant molecules. Nuclear factor-erythroid-2-related factor 2 (Nrf2) is the master regulator of the genes encoding many antioxidant and phase II detoxifying enzymes and related proteins including superoxide dismutase (SOD), catalase, heme oxygenase-1, glutamate cysteine ligase, glutathione transferase, glutathione peroxidase, UDP-glucuronosyltransferase, and NADPH:quinone oxidoreductase-1 among others (Li et al., 2008). Nrf2 is held as an inactive complex bound to a repressor molecule known as Keap1 (Kelch-like erythroid cell-derived-associated protein 1), which facilitates its ubiquitination. Keap1 contains several reactive cysteine residues that serve as sensors of the intracellular redox state. Oxidative or covalent modification of thiols in these cysteine residues or phosphorylation of Nrf2 by protein kinases result in dissociation of Nrf2 from Keap1 and its migration and binding to the antioxidant response elements in the promoter regions of genes encoding the antioxidant and phase 2 detoxifying enzymes (Jeong et al., 2006; Copple et al., 2008). This process is mediated by the heterodimerization of Nrf2 with other transcription factors, such as small Maf, within the nucleus. In addition, Nrf2 can be activated by 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 (Surh et al., 2008).

The Nrf2-mediated regulation of cellular antioxidant and anti-inflammatory machinery plays an important role in the defense against oxidative stress (Li et al., 2008). This is evidenced by the observation that disruption of Nrf2 in mice diminishes or abrogates the induction of these antioxidant genes, indicating their Nrf2-dependent regulation. Furthermore, Nrf2 gene ablation exacerbates diabetes-induced inflammation, oxidative stress, and renal injury and causes a lupus-like autoimmune nephritis in the experimental animals (Yoh et al., 2001, 2008). In a more recent study, we found that severe oxidative stress and inflammation are paradoxically associated with impaired Nrf2 activation and down-regulation of its downstream gene products in the remnant kidneys of five of six nephrectomized rats (Kim and Vaziri, 2010). Based on these observations we concluded that impaired Nrf2 activation contributes to the severity of oxidative stress and inflammation and progression of renal disease in animals with renal mass reduction. The present study was designed to test the hypothesis that oxidative stress and renal inflammation in the Imai rats with spontaneous progressive focal glomerulosclerosis may be accompanied by impaired Nrf2 activation and that attenuation of oxidative stress and inflammation with AT1 receptor blockade in this model may be associated with the restoration of Nrf2 activation.

### TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Proteinuria (mg/24 h)</th>
<th>Creatinine clearance (ml/min/kg body weight)</th>
<th>Serum albumin (g/dl)</th>
<th>Serum creatinine (mg/dl)</th>
<th>Serum urea nitrogen (mg/dl)</th>
<th>Systolic blood pressure (mm Hg)</th>
<th>Serum total-cholesterol (mg/dl)</th>
<th>Serum triglyceride (mg/dl)</th>
<th>Body weight (g)</th>
<th>Kidney weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD Control (n = 6)</td>
<td>18.3 ± 1.4</td>
<td>9.7 ± 1.3</td>
<td>3.58 ± 0.02</td>
<td>0.33 ± 0.01</td>
<td>15.60 ± 0.60</td>
<td>94.75 ± 5.25</td>
<td>68.40 ± 4.20</td>
<td>52.40 ± 8.49</td>
<td>652.80</td>
<td>3.42 ± 0.07</td>
</tr>
<tr>
<td>Imai (n = 6)</td>
<td>571.4 ± 41.7**</td>
<td>2.5 ± 0.4**</td>
<td>2.54 ± 0.04**</td>
<td>1.60 ± 0.40**</td>
<td>72.80 ± 9.32**</td>
<td>179.25 ± 4.52**</td>
<td>499.00 ± 26.90**</td>
<td>765.20 ± 62.66**</td>
<td>518.80 ± 20.17**</td>
<td>6.45 ± 0.38**</td>
</tr>
<tr>
<td>Imai + ARB (n = 6)</td>
<td>18.2 ± 1.6**</td>
<td>8.5 ± 0.5**</td>
<td>3.86 ± 0.08**</td>
<td>0.30 ± 0.01**</td>
<td>14.80 ± 0.37**</td>
<td>95.90 ± 2.11**</td>
<td>118.80 ± 2.97**</td>
<td>118.80 ± 28.17**</td>
<td>541.60 ± 3.25</td>
<td>3.77 ± 0.66**</td>
</tr>
</tbody>
</table>

** p < 0.01 vs. CTL group; ** p < 0.01 vs. untreated Imai group.
Materials and Methods

Animals. Male Imai and Sprague-Dawley rats were obtained from Takeda Clinical Industries (Osaka, Japan). At 10 weeks of age the Imai rats were randomized to ARB-treated (olmesartan, 10 mg/kg/day by gastric gavage for 24 weeks) or vehicle-treated groups. Sprague-Dawley rats served as controls. Olmesartan was obtained from Sankyo Pharmaceutical Inc. (Tokyo, Japan). The given dosage (olmesartan, 10 mg/kg/day) was chosen based on earlier studies that had demonstrated optimal renoprotective and antioxidant effects of this agent in the rat (Kadowaki et al., 2009). The animals were fed regular rat chow and water ad libitum. Arterial blood pressure was determined by tail cuff plethysmography as detailed previously (Vaziri et al., 2007), and timed urine collections were obtained using metabolic cages. The study protocol was approved by the Animal Care and Ethical Committee of the Saga Medical School, Saga, Japan.

TABLE 2
Glomerulosclerosis index (GS), tubulointerstitial injury index (TI), and T lymphocyte (CD5+) and macrophage (ED1+) counts in the kidneys of the 34-week-old Sprague Dawley control rats, untreated Imai rats, and ARB-treated Imai rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n = 6)</th>
<th>Imai (n = 6)</th>
<th>Imai + ARB (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS index</td>
<td>2.08 ± 1.40</td>
<td>235.0 ± 62.89*</td>
<td>10.25 ± 6.19**</td>
</tr>
<tr>
<td>TI damage score</td>
<td>0.61 ± 0.70</td>
<td>4.0 ± 1.92*</td>
<td>0.88 ± 0.61**</td>
</tr>
<tr>
<td>CD5+ cells/TI mm²</td>
<td>7.31 ± 2.92</td>
<td>57.5 ± 15.63*</td>
<td>29.6 ± 10.32**</td>
</tr>
<tr>
<td>ED1+ cells/TI mm²</td>
<td>5.0 ± 1.77</td>
<td>33.3 ± 14.84*</td>
<td>15.6 ± 3.83**</td>
</tr>
</tbody>
</table>

* p < 0.01 vs. control group; **, p < 0.01 vs. untreated Imai group.

Blood and Urine Analysis. Plasma creatinine, urea nitrogen, cholesterol, albumin, and triglycerides were measured with a Synchro CX3 AutoAnalyzer (Beckman Coulter, Inc., Fullerton, CA). Urine protein was quantified by a kit purchased from Wako Pure Chemicals (Tokyo, Japan). Proteinuria was determined in 24-h urine collections, and creatinine clearance was calculated and expressed in absolute values and as milliliters/minute/kilogram of body weight.

Thiobarbituric Acid-Reactive Substance Levels. Renal tissue thiobarbituric acid reactive substance (TBARS) was assayed as described previously (Mihara and Uchiyama, 1978). In brief, 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 (800 and 12,000 g at 4°C for 15 min) as described previously (Johnson and Lardy, 1967; Jung and Pergande, 1985), 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 nanomole of malondialdehyde/milligram of protein by a calibration curve constructed from standard malondialdehyde (0–100 nmol/ml) in 1,1,3,3-tetramethoxypropane. Protein level was evaluated by the micro-biuret method using bovine serum albumin as the standard (Itzhaki and Gill, 1964).

Preparation of Kidney Homogenates and Nuclear Extracts. All solutions, tubes, and centrifuges were maintained at 0 to 4°C. The nuclear extract was prepared as described previously (Sakurai et al., 1996). In brief, 100 mg of kidney cortex was homogenized using a glass-Teflon homogenizer in 0.5 ml of buffer A containing 10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM EGTA.

Fig. 2. Representative photomicrographs of renal sections of untreated Imai rats (A, C, and E) and ARB-treated Imai rats (B, D, and F). A and B, a glomerulus showing sclerosis occupying 50% of the glomerular tuft in an untreated Imai rat (A) in contrast with essentially normal glomerulus in the ARB-treated group (B) (periodic acid-Schiff staining). C and D, tubulointerstitial lymphocyte infiltration is shown in untreated rats (C) and ARB-treated rats (D). E and F, angiotensin-II positive staining is present in proximal tubular cells and in infiltrating cells (arrowheads) in the untreated Imai rats (E) but rarely present in ARB-treated Imai rats (F). Original magnification, 400× in all except E (200×).
mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 mM P-aminobenzamidine using a tissue homogenizer for 20 s. Homogenates were kept on ice for 15 min and then 125 μl of a 10% Nonidet P-40 solution was added and mixed for 15 s, 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% Nonidet P-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 dithiostreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol], mixed for 20 min, and centrifuged for 5 min at 12,000 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 AT1, AT2, NFκB p65, MCP-1, COX-2, Nrf2, Keap1, HO-1, GCLC, and GCLM. Those antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against 12-LO (Cayman Chemical, Ann Arbor, MI), Cu,Zn-SOD and catalase (EMD Chemicals, Inc., Gibbstown, NJ), and Mn-SOD (Millipore Corporation, Billerica, MA), phospho-IκB-α (Cell Signaling Technology, Inc., Danvers, MA), gp91 phox, and Rac1 (BD Biosciences, San Jose, CA) were purchased from the cited sources. Antibodies to histone H1 (Santa Cruz Biotechnology Inc.) and GAPDH (Imgenex, San Diego, CA) were used for measurements of the housekeeping proteins for nuclear and cytosolic target proteins, respectively.

In brief, aliquots containing 50 μg of proteins were fractionated on 8% and 4 to 20% Tris-glycine gel (Novex, San Diego, CA) at 120 V for 2 h and transferred to Hybond-ECL membrane (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). 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 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 (GE Healthcare) at 1:1000 dilution. The membrane was washed four times and developed by autoradiography using the ECL chemiluminescent agents (GE Healthcare). In some instances the membrane was stripped and reprobed for additional proteins.

**Histology and Immunohistology.** Light microscopy studies were performed in formalin-fixed renal sections stained with periodic acid-Schiff and hematoxylin-eosin. The sections were examined by B.R.-I.) in a blinded fashion. Glomerulosclerosis was graded from 0 (normal) to 4+ (sclerosis in more than 75% of the glomerular tuft) as described in previous investigations (Rodríguez-Iturbe et al., 2005; Quiroz et al., 2008). Tubulointerstitial damage was graded according to the extent (percentage) of tubular damage (infiltration, fibrosis, tubular dilatation/atrophy) in successively evaluated fields in the renal cortex.

Lymphocytes, macrophages, and angiotensin II-positive cells were identified using an immunoperoxidase technique as described previously (Rodríguez-Iturbe et al., 2005; Quiroz et al., 2008). CD5+ cells, ED1-positive cells, and angiotensin II-positive cells were identified in glomeruli (positive cells/glomerular cross-section) and tubulointerstitial regions (positive cells/mm²). Monoclonal anti-CD5 and anti-ED-1 (Biosource International, Camarillo, CA) were used to identify lymphocytes and macrophages, respectively. Angiotensin II-positive cells were identified with a rabbit antibody to human angiotensin II (Peninsula Laboratories, Belmont, CA). Secondary antibodies were rabbit anti-mouse and donkey anti-rabbit antibodies with minimal cross-reactivity to rat serum proteins (Accurate Chemical & Scientific, Westbury, NY).

**Data Analysis.** Analysis of variance, multiple range tests, and regression analysis were used in statistical analysis of the data. Data are presented as mean ± S.E. p values < 0.05 were considered significant.

**Results**

**General Data.** Data are summarized in Table 1 and Fig. 1. Body weight in the ARB-treated Imai group was comparable with that of the placebo-treated counterpart and significantly lower in both than in the Sprague-Dawley control rats. In contrast, the kidney weight in the untreated Imai group was significantly higher than those of the Sprague-Dawley group and ARB-treated Imai rats. Plasma creatinine, blood urea nitrogen, urine protein excretion, and blood pressure were significantly elevated, whereas creatinine clearance and plasma albumin were significantly reduced in the untreated Imai rats. These abnormalities were prevented with long-term ARB administration.

**Renal Histology.** In confirmation of our previous study (Rodríguez-Iturbe et al., 2004), the Imai group exhibited significant glomerulosclerosis, tubulo-interstitial injury, and heavy mononuclear cell infiltration in the kidney (Table 2 and Fig. 2). Compared with the control group, the untreated Imai group exhibited a 10-fold increase in the number of...
angiotensin II-positive cells in tubulointerstitial areas. ARB administration resulted in a significant reduction of angiotensin II-positive cells in the treated group (Figs. 1 and 2A).

**AT1 and AT2 Receptor Protein Abundance.** Data are shown in Fig. 3. Compared with the Sprague-Dawley group, the untreated Imai group had significant elevation of AT1 and AT2 receptor protein abundance in the kidney. Up-regulation of AT1 and AT2 receptors in the untreated Imai rats was coupled with marked elevation of the number of angiotensin-positive cells. Long-term therapy with AT1 receptor blocker reversed up-regulation of angiotensin receptors and significantly reduced the number of angiotensin II-positive cells in the treated Imai rats.

**Markers and Mediators of Oxidative Stress.** Data are shown in Fig. 4. Compared with the Sprague-Dawley rats the untreated Imai group exhibited significant elevation of lipid peroxidation products (TBARS) in the renal tissue homogenate and in isolated mitochondrial fraction, pointing to the presence of oxidative stress. This was associated with a significant increase in protein abundance of the gp91phox and Rac1 subunits of NAD(P)H oxidase, which is a major ROS-generating enzyme. Long-term treatment with AT1 receptor blocker significantly reduced accumulation of TBARS and expression of the NAD(P)H oxidase subunits in the kidneys of the study animals.

**Inflammatory Pathways.** Data are illustrated in Figs. 5 and 6. Compared with the Sprague-Dawley group, the untreated Imai rats showed a significant increase in MCP-1, 12-LO, and COX-2 abundance in the kidney. This was associated with a significant increase in the phosphorylated inhibitor of NF-κB and elevated nuclear contents of p65 active subunits of NF-κB, which is the master regulator of numerous proinflammatory and profibrotic cytokines and other mediators. Long-term administration of AT1 receptor blocker prevented these abnormalities.

**Nrf2/Keap1 Pathway.** Data are shown in Figs. 7 and 8. Compared with the Sprague-Dawley rats, the untreated Imai group showed significant reduction of nuclear Nrf2 abundance and a significant increase in cytoplasmic Keap1 abundance in the renal tissue. This was associated with significant reduction of renal tissue HO-1, GCLC, GCLM, catalase, Cu,Zn-SOD, and Mn-SOD protein abundance in the kidney. Long-term ARB administration restored nuclear Nrf2 abundance and expression of its downstream gene products. These findings points to the contribution of Nrf2-Keap1 dysfunction to the pathogenesis of oxidative stress in this model and the role of the angiotensin system in this process.
Discussion

At 34 weeks of age the untreated Imai rats exhibited heavy proteinuria, hyperlipidemia, hypertension, azotemia, reduced creatinine clearance, glomerulosclerosis, tubulointerstitial injury, and inflammation. This was associated with oxidative stress, increased numbers of angiotensin-positive tubular and interstitial cells, and up-regulation of AT1 and AT2 receptors, pointing to the activation of intrarenal angiotensin system in this model. Binding of angiotensin II to AT1 receptor stimulates production of ROS via protein kinase C-mediated activation of NAD(P)H oxidase (Garrido and Griendling, 2009). Under physiological conditions the ROS produced after AT1 receptor activation serve as signaling molecules to mediate regulation of vascular tone and a number of other biological functions of angiotensin II. However, pathological activation of angiotensin system leads to sustained up-regulation of the superoxide-producing enzyme, NAD(P)H oxidase, causing oxidative stress. Oxidative stress, in turn, provokes activation of the NF-κB, transforming growth factor-β, and signal transducer and activator of transcription 3 pathways (Pueyo et al., 2000; Pantano et al., 2006), which results in inflammation, mesangial expansion, podocyte apoptosis, tubular injury, glomerulosclerosis, interstitial fibrosis, NO depletion, and endothelial dysfunction, leading to proteinuria hypertension and progressive deterioration of renal function (Lieberthal et al., 1998; Allen et al., 2003; Bhaskaran et al., 2003; Schmid et al., 2006; Sachse and Wolf, 2007). Thus, angiotensin II can activate proinflammatory pathways by increasing production of ROS. In fact, severe glomerulosclerosis and tubulointerstitial lesions in the untreated Imai rats were accompanied by oxidative stress and up-regulation of NAD(P)H oxidase, activation of NF-κB, increased expression of MCP-1, COX-2, and 12-LO, and heavy infiltration of monocytes and T lymphocytes in the kidney.

It is noteworthy that in addition to exhibiting up-regulation of AT1 receptor the Imai rats showed significant up-regulation of AT2 receptor in the renal tissue. Similar observations have been previously reported in other animal models of kidney disease (Wolf et al., 1997; Esteban et al., 2003). These observations suggest that, in addition to AT1 receptor, AT2 receptor activation may participate in the pathogenesis of angiotensin II-induced activation of the inflammatory and oxidative pathways. This supposition is supported by earlier studies that have demonstrated that angiotensin II-mediated NF-κB activation occurs not only via AT1 receptor activation but also AT2 receptor activation (Ruiz-Ortega et al., 2000; Sadoshima, 2000) and that selective blockade of AT2 receptor can reduce the number of inflammatory cells in the renal tissue in animal models of progressive kidney disease (Wolf et al., 1997; Esteban et al., 2003), pointing to the detrimental role of AT2 receptor in the diseased kidney.

The Nrf2-mediated regulation of cellular antioxidant and anti-inflammatory machinery plays a critical part in the

![Fig. 6. Representative Western blots and group data depicting protein abundance of phospho-IκB (A) and the nuclear contents of the p65 active subunit of NF-κB (B) from renal cortex in the control group and the untreated Imai and ARB-treated Imai groups (n = 6 in each group). Data are presented as mean ± S.E.*, p < 0.05 versus control group; †, p < 0.05 versus Imai group.](image)

![Fig. 7. Representative Western blots and group data depicting nuclear translocation of Nrf2 (A) and protein abundance of its repressor, Keap1 (B) from renal cortex in the control group and the untreated Imai and ARB-treated Imai groups (n = 6 in each group). Data are presented as mean ± S.E.*, p < 0.05 versus control group; †, p < 0.05 versus Imai group.](image)
defense against oxidative stress-induced cytotoxicity and tissue injury (Li et al., 2008). Despite severe oxidative stress and inflammation, which should have caused Nrf2 activation, nuclear Nrf2 content and expression of its downstream gene products were significantly reduced in the untreated Imai rat kidneys. These observations illustrate the contribution of impaired Nrf2 activity to the pathogenesis of oxidative stress, inflammation, and progressive nephropathy in this model. Thus, up-regulation of ROS-producing pathways and deficient Nrf2 activity can work in concert to accentuate oxidative stress, inflammation, and progression of nephropathy in this model. The mechanism responsible for paradoxical impairment of Nrf2 activation despite the prevailing oxidative stress in the advanced phase of nephropathy in this model is uncertain and requires further investigation.

Long-term treatment of the Imai rats with an AT1 receptor blocker initiated before the onset of frank nephropathy (8 weeks of age) prevented the development of kidney disease and hypertension, lowered the number of interstitial angiotensin II-positive cells, and abrogated up-regulations of angiotensin receptors and oxidative and inflammatory pathways in the renal tissue. Preservation of renal function and structure was associated with restoration of Nrf2 activity and expression of its downstream antioxidant and cytoprotective gene products in the renal tissue. These observations illustrate that in addition to reversing angiotensin II-mediated vasoconstriction and attenuating glomerular and systemic hypertension, angiotensin blockade exerts potent antioxidant and anti-inflammatory effects. In this context earlier studies have demonstrated that rennin-angiotensin system blockade suppresses AT1-mediated NF-κB activation (Benigni et al., 2009; Manrique et al., 2009), and the therapeutic benefits of angiotensin system blockade are mediated largely by its antioxidant and anti-inflammatory actions (Kadowaki et al., 2009), which complement its antihypertensive properties.

We note that the absence of serial histological and biochemical data at different stages of development and treatment of the disease precludes identification of the sequence of changes in the oxidative and inflammatory pathways and Nrf2 activity in this model. Serial studies are needed to identify the primary driving factors involved in the observed pathobiological processes and the precise mechanisms of the salutary actions of AT1 receptor blockade in this model.

Heavy glomerular proteinuria causes severe hyperlipidemia in humans and experimental animals (Vaziri, 2003), and hyperlipidemia accelerates progression of renal disease (Vaziri, 2010). The untreated Imai rats develop severe progressive hyperlipidemia, which is accompanied by heavy proteinuria and progressive glomerulosclerosis. We believe that hyperlipidemia in this model is largely caused by the heavy proteinuria. This supposition is based on the observation that treatment of renal disease with ARB results in marked attenuation of hyperlipidemia, whereas lipid lowering therapy is far less effective in ameliorating either progression of renal disease or hyperlipidemia in this model (Rodriguez-Iturbe et al., 2004). However, this supposition has not been definitively proven and awaits further exploration.

In conclusion, progressive kidney disease in the Imai rats is associated with up-regulation of the intrarenal angiotensin system, oxidative stress, inflammation, and conspicuous impairment of Nrf2 activation. Long-term therapy with an AT1 receptor blocker prevents the development of nephropathy, oxidative stress, and inflammation and restores Nrf2 activity in this model. Thus AT1 receptor blockade attenuates oxidative stress by simultaneously lowering production of ROS and raising production of endogenous antioxidants. The precise mechanism by which AT1 receptor blocker restored Nrf2 activity in this model is unknown and awaits future investigation.

Authorship Contributions

* Participated in research design: Vaziri.
* Conducted experiments: Kim, Sato, and Rodriguez-Iturbe.
* Performed data analysis: Kim, Sato, and Rodriguez-Iturbe.
* Wrote or contributed to the writing of the manuscript: Vaziri.

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