HHS Public Access

Author manuscript

Kidney Int. Author manuscript; available in PMC 2014 August 01.

Published in final edited form as:

Kidney Int. 2014 February ; 85(2): 333–343. doi:10.1038/ki.2013.343.

Nrf2 suppresses lupus nephritis through inhibition of oxidative injury and the NF-κB-mediated inflammatory response

Tao Jiang[#1,2,*], **Fei Tian**[#3], **Hongting Zheng**[4], **Samantha A. Whitman**[1], **Yifeng Lin**[2], **Zhigang Zhang**[2], **Nong Zhang**[2], and **Donna D. Zhang**[1,*]

[1]Department of Pharmacology and Toxicology, University of Arizona, Tucson, Arizona

[2]Department of Pathology, Shanghai Medical College, Fudan University, Shanghai, China

[3]Department of Medical Microbiology and Parasitology, Institutes of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai, China

[4]Department of Endocrinology, Xinqiao Hospital, Third Military Medical University, Chongqing, China

[#] These authors contributed equally to this work.

Abstract

The generation of reactive oxygen species plays a pivotal role in both acute and chronic glomerular injuries in patients with lupus nephritis. Since the transcription factor Nrf2 is a major regulator of the antioxidant response and is a primary cellular defense mechanism we sought to determine a role of Nrf2 in the progression of lupus nephritis. Pathological analyses of renal biopsies from patients with different types of lupus nephritis showed oxidative damage in the glomeruli, accompanied by an active Nrf2 antioxidant response. A murine lupus nephritis model using Nrf2+/+ and Nrf2−/− mice was established using pristine injection. In this model, Nrf2−/− mice suffered from greater renal damage and had more severe pathological alterations in the kidney. In addition, Nrf2+/+ mice showed ameliorative renal function when treated with sulforaphane, an Nrf2 inducer. Nrf2−/− mice had higher expression of TGFβ1, fibronectin and iNOS. In primary mouse mesangial cells, the nephritogenic monoclonal antibody R4A activated the NF-κB pathway and increased the level of reactive oxygen species, iNOS, TGFβ1 and fibronectin. Knockdown of Nrf2 expression aggravated all aforementioned responses induced by R4A. Thus, these results suggest that Nrf2 improves lupus nephritis by neutralizing reactive oxygen species and by negatively regulating the NF-κB and TGFβ1 signaling pathways.

Keywords

lupus nephritis; Nrf2; ROS; NF-κB; TGFβ1; iNOS

[*]Address correspondence and reprint requests to Donna D. Zhang, Ph.D, Department of Pharmacology and Toxicology, University of Arizona, 1703 E Mabel St, Tucson AZ 85721, dzhang@pharmacy.arizona.edu; Tao Jiang, Department of Pharmacology and Toxicology, University of Arizona, 1703 E Mabel St, Tucson AZ 85721, tjiang@pharmacy.arizona.edu.

Disclosure

All the authors declare no conflict of interest.

Lupus nephritis is a severe autoimmune disease associated with a high rate of morbidity and mortality. A large body of evidence indicates that production of autoantibodies and glomerular immune complex deposition are the initial events in the pathogenesis of the disease[1-4]. The deposition of the immune complex triggers a cascade of events in the inflammatory response that are accompanied by the generation of reactive oxygen species (ROS), which play a pivotal role in both acute and chronic glomerular injuries in lupus nephritis patients[5, 6]. Detection of lipid oxidation, oxidative DNA damage, and protein oxidation in lupus patients provide strong evidence for the involvement of ROS in this disease[7-9]. Moreover, several mechanisms by which ROS promotes acute and chronic tissue damage in lupus nephritis have been described in detail [10].

Nrf2 is a major regulator of the antioxidant response and is a primary cellular defense mechanism[11, 12]. It regulates target genes encoding intracellular antioxidants, phase II detoxifying enzymes, and other effectors that promote cell survival and maintain cellular redox homeostasis[13]. Nrf2 up-regulates its target genes through an antioxidant response element (ARE) in the regulatory regions of these genes in response to oxidative stress[14]. The essential role of Nrf2 in combating oxidative stress has been clearly demonstrated by the findings that Nrf2-null ($Nrf2^{-/-}$) mice have increased sensitivity to a variety of perturbations[12, 14, 15]. Considering the important role of ROS in the pathogenesis of lupus nephritis, we hypothesize that Nrf2 protects against renal injury by neutralizing ROS and thus reducing tissue damage. Additionally, several studies show that some Nrf2 inducers can inhibit the activation of NF-κB pathway[16-18], a redox sensitive transcription factor. However, the interaction between these two pathways in lupus nephritis remains unclear.

In this study, we investigated the role of Nrf2 in lupus nephritis. As the pivotal pathway for redox homeostasis, we hypothesize that Nrf2 is essential to maintain renal function and antagonize renal damage during the progression of lupus nephritis.

RESULTS

The glomeruli of human LN patients are under oxidative stress and exhibit increased Nrf2 expression

A total of 60 human kidney biopsies were collected, 48 cases were from lupus patients and 12 cases were from healthy people. Compared to normal glomeruli, the glomeruli of lupus nephritis patients showed expansion of mesangium, increased cellularity, fibrinoid necrosis and thickening of capillary walls (Fig.1, compare panel A to panel B-F). Deposition of immunoglobulin within the glomeruli was seen in all classes (Fig.1, panel H-L). Nrf2 was barely expressed in normal glomeruli (Fig.1, panel M), whereas the expression was elevated in lupus patients (Fig.1, panel N-R). Consistent with these results, the Nrf2 downstream gene NAD(P)H dehydrogenase, quinone 1(NQO1) was also upregulated in the glomeruli of lupus nephritis patients, confirming the activation of the Nrf2 pathway. (Fig.1, compare panel S to panel T-X). DNA damage induced by oxidative stress was measured using an antibody against 8-Oxo-dG. Positive nuclear staining was detected in the tissues from lupus nephritis patients, but not in the normal kidney tissues (Fig.1, compare panel Y to panel Z;D'). To obtain a quantitative analysis of Nrf2, NQO1, and 8-Oxo-dG expression, the

glomeruli from each section were analyzed by i-Solution software. The average expression of Nrf2, NQO1 and 8-Oxo-dG are shown as a percentage in Figure 1 (Fig 1, panel E'). Compared to the normal kidney, the expression of Nrf2, NQO1 and 8-Oxo-dG were significantly higher in the glomeruli from any class of lupus nephritis (Fig 1, panel E', * $P<0.05$, ** $P<0.05$). However, the expression of Nrf2, NQO1 and 8-Oxo-dG did not correlate with the severity of lupus nephritis, since type III but not type IV, which is the severest form of lupus nephritis, had the highest expression of Nrf2. Taken together, these data indicate that the glomeruli of lupus nephritis patients experience oxidative stress, which triggers the activation of the Nrf2-mediated antioxidant response. The expression of Nrf2 was also analyzed in other representative types of nephritis; acute proliferative glomerulonephritis, IgA nephropathy and purpuric nephropathy demonstrated positive Nrf2 expression (Fig 1, panel F'). These findings suggest that the increased activation of Nrf2 in glomeruli from different types of nephritis is a general phenomena resulting from immune-complex deposition.

Renal damage induced by pristane treatment is more severe in $Nrf2^{-/-}$ mice

To further explore the role of Nrf2 in the progression of lupus nephritis, a pristane-induced murine model was employed. During the course of the experiment, two $Nrf2^{-/-}$ mice in the pristane treatment group died at the 21st and 23rd week, respectively. Nevertheless, the survival rate between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ groups showed no significant difference (Fig. 2A). The spleen to body weight ratio, however, was significantly increased in the pristane-treated groups, regardless of the genotype (Fig. 2B). In addition, $Nrf2^{-/-}$ mice had a significantly higher spleen to body weight ratio than $Nrf2^{+/+}$ mice in both untreated and treated groups (Fig. 2B, # $P<0.05$). UACR, an indicator of kidney vascular permeability, was measured every four weeks following pristane injection to show any alterations in renal functions. Pristane markedly increased UACR in a time-dependent manner in both genotypes (Fig. 2C, * $P<0.05$). $Nrf2^{-/-}$ mice however showed trends toward a higher UACR than $Nrf2^{+/+}$ mice starting at 16 weeks that reached its apex at the end of the study (28 week post-pristane injection) (Fig. 2C, # $P<0.05$). The sera from the mice were collected and used to measure auto-antibodies titers at a 1:50 dilution. Anti-ssDNA, anti-nRNP, and anti-Histone Ig's were significantly increased after pristane treatment (Fig. 2D-F). $Nrf2^{-/-}$ mice had higher levels of anti-ssDNA antibodies with or without treatment of pristane (Fig. 2D, #$P<0.05$). Treatment of pristane induced the deposition of IgG and IgM within glomeruli in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice (Fig. 2G, H). In addition, another lupus model was set using $Nrf2^{+/+}$ mice only to study the protective role of sulforphane on lupus nephritis. Although the administration of sulforaphane did not significantly affect the body to spleen weight ratio (Supplementary Fig.1A), it ameliorated the renal function (Supplementary Fig.1B, # $P<0.05$). Taken together, these results indicate that $Nrf2^{-/-}$ mice suffer greater renal damage after pristane treatment and that the increased Nrf2 level can ameliorate these complications, implicating the essential role of Nrf2 in protecting against pristane-induced lupus nephritis.

Oxidative DNA damage and elevated Nrf2 expression are observed in the glomeruli of a lupus nephritis mice model

Glomerular lesions, such as mesangial cell proliferation, mesangium expansion, and infiltration of inflammatory cells were observed in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice following pristane injection (Fig.3, panel A-D). One notable observation is that untreated $Nrf2^{-/-}$ mice displayed a similar degree of glomerular damage as the pristane-treated wild-type mice (Fig. 3, compare panel B with C). Pristane treatment induced Nrf2 expression in glomeruli of $Nrf2^{+/+}$ mice (Fig.3, compare panel E with F). As expected, Nrf2 was not detected within glomeruli in $Nrf2^{-/-}$ mice (Fig. 3, panel G and H). Furthermore, treatment with pristane increased NQO1 expression in $Nrf2^{+/+}$ mice (Fig.3, compare panel I with J). Although NQO1 was not detected in the untreated $Nrf2^{-/-}$ mice, it was detected in some glomeruli of pristane-treated $Nrf2^{-/-}$ mice, indicating the possible activation of NQO1 by an Nrf2 independent pathway (Fig. 3, compare panel K with L). $Nrf2^{-/-}$ mice were more sensitive to pristane-induced oxidative DNA damage than $Nrf2^{+/+}$ mice (Fig.3, compare panel N with P). Interestingly, $Nrf2^{-/-}$ mice displayed higher levels of oxidative damage even in the untreated condition (Fig.3, compare panel M with O), suggesting that basal levels of Nrf2 are essential to protect cells from DNA damage. In addition, administration of sulforaphane ameliorated the pathological alterations in glomeruli of $Nrf2^{+/+}$ mice induced by pristane (Supplementary Fig. 2C-F). Moreover, sulforaphane induced activation of Nrf2 and inhibited the deposition of fibronectin and production of 8-Oxo-dG within glomeruli (Supplementary Fig. 2G-V). The pathological alterations were assessed and scored (Fig. 3R, Supplementary Fig. 2B). Although, there is a trend some pathology endpoints were not significantly protected by sulforaphane. All stained sections were analyzed by i-Solution software (Fig 3, panel Q, * $P<0.05$ vs. control; # $P<0.05$ vs. Nrf2 +/+ mice). Similarly, sulforaphane ameliorated the oxidative stress and deposition of extracellular matrix (Supplementary Fig. 2A). Collectively, these results demonstrate that Nrf2 is essential in protecting against both basal and pristane-induced renal injuries.

Increased expression of TGFβ1, fibronectin and iNOS in $Nrf2^{-/-}$ mice, compared to $Nrf2^{+/+}$ mice

The mRNA level of key factors of glomerulosclerosis, transforming growth factor β1 (TGFβ1) and fibronectin, were measured in order to explore the role of Nrf2 in lupus neprhitis. Consistent with our previous report[19], the basal mRNA level of TGFβ1 was higher in $Nrf2^{-/-}$ mice than $Nrf2^{+/+}$ mice (Fig. 4A, TGFβ1 panel). Similarly, the level of fibronectin was also elevated in $Nrf2^{-/-}$ mice (Fig. 4A, FN panel). In response to pristane treatment, the mRNA level of TGFβ1 and fibronectin was markedly increased in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice (Fig. 4A, TGFβ1 and FN panel). The inflammation associated enzyme, iNOS also showed similar expression pattern with TGFβ1 (Fig. 4A, iNOS panel). Sulforaphane suppressed the mRNA level of TGFβ1, fibronectin and iNOS after pristane treatment, but induced the transcription of NQO1 (Supplementary Fig. 1C-F). As expected, the protein level of NQO1 was increased by pristane treatment in $Nrf2^{+/+}$, but not in $Nrf2^{-/-}$ mice (Fig. 4B), implicating the existence of oxidative stress in the murine lupus nephritis model. The protein level of fibronectin was increased after treatment of pristane, and it was higher in $Nrf2^{-/-}$ mice than $Nrf2^{+/+}$ mice (Fig. 4B, C). In addition, the protein level of Nrf2 and NQO1 was induced in $Nrf2^{+/+}$ mice with administration of sulforaphane

(Supplementary Fig.1G). More importantly, the inflammation-related pathway, NF-κB showed increased activation in $Nrf2^{-/-}$ mice compared to $Nrf2^{+/+}$ mice after pristane treatment (Fig. 4B). Taken together, these data demonstrate that $Nrf2^{-/-}$ mice have excessive expression of TGFβ1, fibronectin, and iNOS after pristane treatment. In addition, the increased activation of NF-κB in $Nrf2^{-/-}$ mice may explain the more severe renal injuries in $Nrf2^{-/-}$ mice and suggests that Nrf2 may negatively regulate NF-κB.

Nrf2 negatively regulates R4A-induced iNOS expression by suppressing the NF-κB signaling pathway

To further confirm the negative effects of Nrf2 on the NF-κB and TGFβ1 pathways, an *in vitro* cell culture system was utilized. Primary mesangial cells were isolated from MRL/*lpr* mice that are prone to developing lupus. The monoclonal antibody R4A, an anti-dsDNA antibody that was found to be nephritogenic, was used to treat the primary mesangial cells. A non-nephritogenic MOPC-141 antibody was included as a negative control. As expected, expression of Nrf2 and NQO1 decreased significantly after Nrf2-siRNA transfection (Fig. 5A, I). Interestingly, knockdown of Nrf2 increased the expression of iNOS. R4A treatment did not change the expression of Nrf2 and NQO1, but increased the expression of iNOS in cells transfected with either control-siRNA or Nrf2-siRNA (Fig. 5A). The basal level of ROS production was minimally changed after Nrf2 knockdown (Fig. 5B). Conversely, when cells were treated with R4A, the ROS level was increased in cells transfected with control-siRNA and increased even more in cells transfected with Nrf2-siRNA (Fig. 5B). R4A had no effect on the Nrf2 mRNA level (Fig. 5C) but induced NQO1 mRNA expression was induced by R4A treatment and this response was abrogated by knocking down of Nrf2 (Fig. 5D). iNOS mRNA level was dramatically induced by R4A treatment (Fig. 5E). Knockdown of Nrf2 increased the mRNA level of iNOS in both MOPC-141- and R4A-treated cells. These data support our *in vivo* findings demonstrating an increase in the iNOS mRNA level in $Nrf2^{-/-}$ mice. Similar to iNOS expression, the mRNA expression of TGFβ1 was negatively regulated by Nrf2 (Fig. 5F). R4A increased TGFβ1 mRNA level that was further increased by Nrf2 knockdown (Fig. 5F).

Given the fact that iNOS is one of the direct target genes of the NF-κB pathway, the effect of Nrf2 on this pathway was measured. Phosphorylation of p65 and IκB was measured to detect activation of the NF-κB signaling pathway. Treatment of cells with R4A resulted in an increase in the level of phosphorylated p65 and IκB whereas the total amount of p65 and IκB remained unchanged (Fig. 5G). Reduction of Nrf2 by transfection of Nrf2-siRNA resulted in a much stronger activation of the NF-κB signaling pathway in response to R4A treatment (Fig. 5G). Next, a κB reporter gene assay was transfected into cells that either overexpress or have Nrf2 knocked down of Nrf2 to further confirm the negative correlation between Nrf2 and the NF-κB pathway. R4A activated the κB-dependent luciferase activity, which was reduced by ectopic expression of Nrf2 in a dose-dependent manner (Fig. 5H), and induced by decreased expression of Nrf2 (Fig. 5I). Collectively, these data indicate that Nrf2 negatively regulates the R4A-induced activation of the NF-κB and TGFβ1 signaling pathways, thus suppressing the expression of the downstream genes of these two pathways.

Inhibition of NF-B pathway alleviated the production of ROS and expression of iNOS induced by R4A

Next, we determined whether the increased production of ROS and expression of iNOS induced by R4A in Nrf2$^{-/-}$ cells is mediated the NF-κB pathway. A NF-κB p65 inhibitory peptide was used to block the R4A triggered NF-κB pathway (Fig. 6A). In the mesangial cells from MRL/*lpr* mice that have Nrf2 knockdown, the R4A-induced production of ROS was blocked by incubating the cells with p65 inhibitory peptide (Fig. 6B, # $P<0.05$). Treatment with R4A significantly induced the mRNA expression of NQO1; however, there was no significant difference between the control and p65 inhibitor treated groups (Fig. 6C).

Although the mRNA level of TGFβ dramatically increased after R4A treatment, incubation with the p65 inhibitory peptide did not affect its expression (Fig. 6E). However, p65 inhibition blocked the transcription of iNOS (Fig. 6D, # $P<0.05$) and also decreased the protein level (Fig. 6F). These data indicated that the NF-κB pathway is important for regulating ROS release and the expression of iNOS triggered by R4A when Nrf2 level are low. They also suggest that increased activation of the NF-κB pathway in Nrf2 deficient mice or cells can account for the more severe renal injuries.

DISCUSSION

Although the Nrf2-ARE signaling pathway has proved to be useful in combating several diseases[20, 21], the role of Nrf2 in lupus nephritis remains unclear. Previous studies have shown that Nrf2$^{-/-}$ mice spontaneously develop lupus-like autoimmune nephritis at 60-weeks [22], These findings suggest that the loss of Nrf2 promotes the development of lupus nephritis, indicating a preventive or protective role for Nrf2. Conflicting data, however, were obtained from the same group when they used a genetic Nrf2$^{-/-}$ *lpr*/*lpr* mouse model[23]. Nrf2$^{-/-}$ *lpr*/*lpr* mice lived longer and showed an improvement in the nephritis manifestation compared to Nrf2$^{+/+}$ *lpr*/*lpr* mice. This study suggests that Nrf2 aggravates lupus. In addition, two other studies demonstrated that Nrf2$^{-/-}$ mice, with the same background that Takahashi's group used, developed a lupus-like autoimmune disease with renal injuries[24, 25]. Thus, the role of Nrf2 in the development of lupus nephritis is still unclear and further studies are needed.

In our study, we demonstrate that Nrf2 protects against lupus nephritis by inhibiting the activation of the NF-κB pathway and deposition of extracellular matrix. First, the lupus nephritis patients manifested increased oxidative stress in kidney, as consistent with the previous report by Moroni G et al [26]. Despite the increased level of Nrf2, NQO1 and 8-Oxo-dG were observed in all types of lupus nephritis, it is more likely a result of compensatory mechanism and may be surpassed by the oxidative stress, and therefore is not able to prevent renal injuries. Moreover, there is no significant correlation between the severity of lupus nephritis and the expression of Nrf2, NQO1 and 8-Oxo-dG. The insufficient case number may account for this finding, since we only had access to 4 and 7 case in Class I and Class V, respectively. It is interesting that Córdova EJ et al reported that the two single nucleotide polymorphisms of Nrf2 are not associated with susceptibility to childhood-onset lupus, but it could contribute to the development of kidney malfunction [27]. This contribution is quite reasonable since modification of Nrf2 may increase oxidative stress and promote lupus

nephritis. Second, in a mouse pristane-induced lupus model, Nrf2$^{-/-}$ mice suffered more renal damages, strengthened the activation of NF-κB pathway, and had more deposition of extracellular matrix than wild-type mice. In addition, sulforaphane, an Nrf2 inducer, prevented the pristane-induced lupus model in wild type mice. Our data confirmed the importance of anti-oxidant stress and NF-κB pathway in lupus, which is consistent with the previous report by Tsai PY et al [28]. In contrast to the previous reports on spontaneous formation of autoimmune nephritis in Nrf2$^{-/-}$ mice, we did not observe autoimmune nephritis-like phenotype in the untreated group. Presumably, this may be due to difference in the length of the individual experiments, i.e., 36 weeks in our investigation versus 12 months in the previous report[24]. Although Ma, *et al*. reported that spontaneous glomerulonephritis could appear as early as the age of 2 months, the large-scale death of Nrf2$^{-/-}$ mice caused by nephritis in those studies was observed at 36 weeks[25].

As our data highlight, the protective effects of Nrf2 on the progression of lupus nephritis come from several aspects, including ROS scavenging, inhibition of the NF-κB pathway and TGFβ1 pathway. The NF-κB pathway is crucial in the development of lupus [29]. It controls expression of several genes involved in inflammatory responses, such as iNOS, which is expressed at high levels in lupus and is correlated with disease severity[30, 31]. In our study, the activation of the NF-κB pathway and expression of iNOS were negatively regulated by Nrf2. Therefore, Nrf2 may inhibit the development of lupus by suppressing the NF-κB-mediated inflammatory response in addition to its ROS scavenging activity.

The crosstalk of these two pathways has been extensively investigated, yet conflicting results still exist. Both positive and negative regulation between Nrf2 and NF-κB has been reported [32]. Certainly chemopreventive compounds can activate Nrf2 while inhibiting the NF-κB pathway and its downstream genes[16, 18, 33]. On the other hand, many agents or conditions have been shown to increase the activity of both Nrf2 and NF-κB [32]. Additionally, Yang *et al.* reported significantly decreased activity of NF-κB in the liver isolated from Nrf2$^{-/-}$ mice and a lower NF-κB binding activity in Nrf2$^{-/-}$ fibroblasts[34]. However, Chen *et al.* reported that overexpression of Nrf2 could repress the expression of NF-κB downstream genes without affecting the activity of NF-κB [35]. Therefore, the existing literature regarding the mutual regulation between Nrf2 and NF-κB is inconsistent and further study is needed to elucidate the molecular mechanism.

As a minor point of this report, we also observed that the Nrf2 may negatively regulate TGFβ1 and fibronectin expression both *in vivo* and *in vitro*, which is consistent with our previous finding in a diabetic mouse model and a human mesangial cell model[19]. Further studies are needed to dissect the detailed mechanisms by which Nrf2 suppresses NF-κB and TGFβ1. In summary, our study clearly demonstrates that Nrf2 protects renal cell from developing lupus nephritis by scavenging ROS and inhibiting the NF-κB and TGFβ1 signaling pathways.

MATERIALS AND METHODS

Patients, renal histology and immunoglobulin deposition

Renal biopsies (n = 60) were obtained from the Department of Pathology, Fudan University, from 2008 to 2011. Paraffin sections (2 μm) were stained with hematoxylin and eosin (H&E). Immunoglobulin deposition was detected by immunofluorescence. Two individual pathologists diagnosed all the cases. According to the WHO classification, 39 cases were categorized into class I, II, III, IV and V of lupus nephritis, and 9 cases into acute proliferative glomerulonephritis, IgA nephropathy and purpuric nephropathy (3 cases of each). In addition, 12 cases of normal kidney with no lupus history were selected as negative control (Table 1). The selected clinical characteristics of patients are shown in supplementary table 1. Permission to use the fixed tissue sections for research purposes was obtained and approved by the Ethics Committee from Fudan University, China.

Animals and treatment

$Nrf2^{-/-}$ mice were originally generated in Dr. Kan's laboratory (University of California, San Francisco). Female mice were intraperitoneally injected once with 0.5 mL pristane (Sigma, MO), phosphate-buffered saline (PBS) at 8 weeks of age and pristine plus sulforaphane (12.5 mg/kg, Santa Cruz, CA) every other day. The mice were euthanized 28-weeks post-injection and kidneys and spleens were isolated and weighed. Kidneys were fixed for histology analysis and frozen for protein and RNA extraction. Female MRL-*lpr*/*lpr* (MRL/*lpr*) mice were purchased from the Institute of Zoology, Chinese Academy of Science for isolation of primary mesangial cells.

Auto-antibodies titers and urine albumin-to-creatinine ratio (UACR) measurement

Freshly voided spot urine samples and blood samples were collected. ELISA kits were used to measure urine albumin and creatinine levels (albumin: Bethyl Laboratories, Houston, TX; creatinine: Exocell, Philadelphia, PA). The UACR ratio is expressed as micrograms of albumin to milligrams of creatinine. ELISA kits were used to measure the auto-antibodies titers (Alpha Diagnostic Intl. Inc., TX). The deposition of antibodies was measured by immunofluorescence.

Immunohistochemistry analysis and detection of oxidative DNA damage

Both renal biopsies and kidney tissues from the animal model were analyzed. Furthermore, the stained results were analyzed by image quantitation software (IMT i-Solution INC., Vancouver, Canada). The information of antibodies used in this study and the methods of image quantitation are described in the supplementary materials. The protocol for detection of 8-Oxo-dG was described previously[19].

qRT-PCR and immunoblot assay

Total RNA from kidney tissues or cells was extracted using Trizol (Invitrogen, CA). Equal amounts of RNA (2 μg) were reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, IN). Taqman probes and primers, and the methods used in this study are described in supplementary material.

Glomerular isolation, primary mesangial cell culture, and antibody treatment

The protocol was followed as described by Dr. Putterman's group[36]. The detailed methods are described in supplementary material. The nephritogenic antibody R4A was a gift form Dr. Betty Diamond [37], and the control antibody MOPC-141 was purchased from Sigma.

Reactive oxygen species detection, siRNA transfection, and luciferase reporter gene assay

The dichlorofluorescein for ROS detection was purchased from Sigma. The validated siRNA was from Qiagen. The dual luciferase assay system is a product of Promega (Promega, WI). A NF-κB p65 inhibitory peptide was purchased from IMGENEX (IMGENEX Corp., CA). The detailed protocols are described in supplementary material.

Statistical analyses

Results are expressed as mean ± standard deviation (SD). Unpaired student t-tests were used to compare the means of two groups. One-way ANOVA (Tukey test) was applied to compare the means of three or more groups. The Wilcoxon (Gehan) statistical test was used to analyze the survival rate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank Dr. Betty Diamond for providing the R4A monoclonal antibody. This work was supported by the following grants: Science and Technology Commision of Shanghai Municipality (11ZR1402400 to T.J.), National Institute of Environmental Health (2R01 ES015010 to D.D.Z.), Nation Cancer Institute (R01 CA154377 to D.D.Z.), and National Natural Science Foundation of China (81228023 to D.D.Z. and H.Z.).

References

1. Cameron JS. Lupus nephritis. J Am Soc Nephrol. 1999; 10:413–424. [PubMed: 10215343]
2. Couser WG. Basic and translational concepts of immune-mediated glomerular diseases. J Am Soc Nephrol. 2012; 23:381–399. [PubMed: 22282593]
3. Kaveri SV, Mouthon L, Bayry J. Basophils and nephritis in lupus. N Engl J Med. 2010; 363:1080–1082. [PubMed: 20825323]
4. Grande JP. Experimental models of lupus nephritis. Contrib Nephrol. 2011; 169:183–197. [PubMed: 21252519]
5. Kovacic P, Jacintho JD. Systemic lupus erythematosus and other autoimmune diseases from endogenous and exogenous agents: unifying theme of oxidative stress. Mini Rev Med Chem. 2003; 3:568–575. [PubMed: 12871159]
6. Nath KA, Fischereder M, Hostetter TH. The role of oxidants in progressive renal injury. Kidney Int Suppl. 1994; 45:S111–115. [PubMed: 8158878]
7. Evans MD, Cooke MS, Akil M, et al. Aberrant processing of oxidative DNA damage in systemic lupus erythematosus. Biochem Biophys Res Commun. 2000; 273:894–898. [PubMed: 10891343]
8. Frostegard J, Svenungsson E, Wu R, et al. Lipid peroxidation is enhanced in patients with systemic lupus erythematosus and is associated with arterial and renal disease manifestations. Arthritis Rheum. 2005; 52:192–200. [PubMed: 15641060]
9. Morgan PE, Sturgess AD, Hennessy A, et al. Serum protein oxidation and apolipoprotein CIII levels in people with systemic lupus erythematosus with and without nephritis. Free Radic Res. 2007; 41:1301–1312. [PubMed: 17957542]

10. Grande JP. Mechanisms of progression of renal damage in lupus nephritis: pathogenesis of renal scarring. Lupus. 1998; 7:604–610. [PubMed: 9884097]
11. Itoh K, Ishii T, Wakabayashi N, et al. Regulatory mechanisms of cellular response to oxidative stress. Free Radic Res. 1999; 31:319–324. [PubMed: 10517536]
12. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol. 2007; 47:89–116. [PubMed: 16968214]
13. Zhang DD. Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev. 2006; 38:769–789. [PubMed: 17145701]
14. Chan K, Han XD, Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci U S A. 2001; 98:4611–4616. [PubMed: 11287661]
15. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol Med. 2004; 10:549–557. [PubMed: 15519281]
16. Ahmad R, Raina D, Meyer C, et al. Triterpenoid CDDO-Me blocks the NF-kappaB pathway by direct inhibition of IKKbeta on Cys-179. J Biol Chem. 2006; 281:35764–35769. [PubMed: 16998237]
17. Karuri AR, Huang Y, Bodreddigari S, et al. 3 H-1,2-dithiole-3-thione targets nuclear factor kappaB to block expression of inducible nitric-oxide synthase, prevents hypotension, and improves survival in endotoxemic rats. J Pharmacol Exp Ther. 2006; 317:61–67. [PubMed: 16371450]
18. Sriram N, Kalayarasan S, Sudhandiran G. Epigallocatechin-3-gallate augments antioxidant activities and inhibits inflammation during bleomycin-induced experimental pulmonary fibrosis through Nrf2-Keap1 signaling. Pulm Pharmacol Ther. 2009; 22:221–236. [PubMed: 19138753]
19. Jiang T, Huang Z, Lin Y, et al. The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. Diabetes. 2010; 59:850–860. [PubMed: 20103708]
20. Zhang DD. The Nrf2-Keap1-ARE signaling pathway: the regulation and dual function of Nrf2 in cancer. Antioxid Redox Signal. 2010
21. Hu R, Saw CL, Yu R, et al. Regulation of Nrf2 Signaling for Cancer Chemoprevention: Antioxidant Coupled with Anti-inflammatory. Antioxid Redox Signal. 2010
22. Yoh K, Itoh K, Enomoto A, et al. Nrf2-deficient female mice develop lupus-like autoimmune nephritis. Kidney Int. 2001; 60:1343–1353. [PubMed: 11576348]
23. Morito N, Yoh K, Hirayama A, et al. Nrf2 deficiency improves autoimmune nephritis caused by the fas mutation lpr. Kidney Int. 2004; 65:1703–1713. [PubMed: 15086909]
24. Li J, Stein TD, Johnson JA. Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice. Physiol Genomics. 2004; 18:261–272. [PubMed: 15173550]
25. Ma Q, Battelli L, Hubbs AF. Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2. Am J Pathol. 2006; 168:1960–1974. [PubMed: 16723711]
26. Moroni G, Novembrino C, Quaglini S, et al. Oxidative stress and homocysteine metabolism in patients with lupus nephritis. Lupus. 2010; 19:65–72. [PubMed: 19933721]
27. Cordova E, Velazquez-Cruz R, Centeno F, et al. The NRF2 gene variant -653G/A is associated with nephritis in childhood-onset systemic lupus erythematosus. Lupus. 2010
28. Tsai PY, Ka SM, Chang JM, et al. Epigallocatechin-3-gallate prevents lupus nephritis development in mice via enhancing the Nrf2 antioxidant pathway and inhibiting NLRP3 inflammasome activation. Free Radic Biol Med. 2011; 51:744–754. [PubMed: 21641991]
29. Okamoto T. NF-kappaB and rheumatic diseases. Endocr Metab Immune Disord Drug Targets. 2006; 6:359–372. [PubMed: 17214582]
30. Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sci. 2004; 75:639–653. [PubMed: 15172174]
31. Wang G, Pierangeli SS, Papalardo E, et al. Markers of oxidative and nitrosative stress in systemic lupus erythematosus: correlation with disease activity. Arthritis Rheum. 2010; 62:2064–2072. [PubMed: 20201076]

32. Wakabayashi N, Slocum SL, Skoko JJ, et al. When NRF2 Talks, Who's Listening? Antioxid Redox Signal. 2010

33. Liu YC, Hsieh CW, Wu CC, et al. Chalcone inhibits the activation of NF-kappaB and STAT3 in endothelial cells via endogenous electrophile. Life Sci. 2007; 80:1420–1430. [PubMed: 17320913]

34. Yang H, Magilnick N, Lee C, et al. Nrf1 and Nrf2 regulate rat glutamate-cysteine ligase catalytic subunit transcription indirectly via NF-kappaB and AP-1. Mol Cell Biol. 2005; 25:5933–5946. [PubMed: 15988009]

35. Chen XL, Dodd G, Thomas S, et al. Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. Am J Physiol Heart Circ Physiol. 2006; 290:H1862–1870. [PubMed: 16339837]

36. Qing X, Zavadil J, Crosby MB, et al. Nephritogenic anti-DNA antibodies regulate gene expression in MRL/lpr mouse glomerular mesangial cells. Arthritis Rheum. 2006; 54:2198–2210. [PubMed: 16804897]

37. Katz JB, Limpanasithikul W, Diamond B. Mutational analysis of an autoantibody: differential binding and pathogenicity. J Exp Med. 1994; 180:925–932. [PubMed: 8064241]

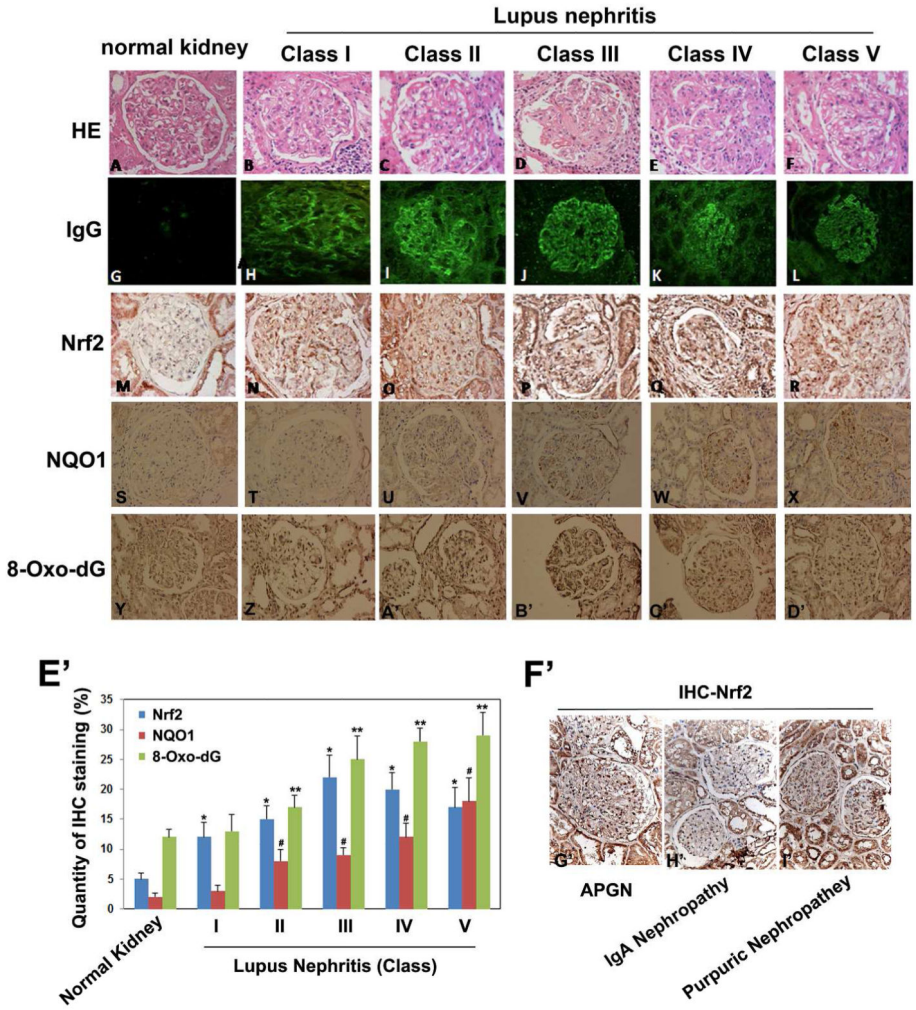

Figure 1. The glomeruli of human lupus nephritis patients are under oxidative stress and exhibit increased Nrf2 expression

Renal biopsy specimens were processed for pathology and immunohistochemistry (IHC) analysis. (Panel A-F) H&-E staining of normal kidney tissues and tissues from lupus nephritis patients. (Panel G-L) Immunofluorescence analysis with an anti-IgG antibody for detecting immunoglobulin deposition. (Panel M-X) IHC analysis with an antibody against Nrf2 or NQO1 for detecting activation of the Nrf2 pathway. (Panel Y to D') IHC analysis with an anti-8-Oxo-dG antibody for detection of oxidative damage. One representative image from either normal renal tissue or each class of lupus nephritis patients is shown (Magnification ×400). (Panel E') The staining of Nrf2, NQO1 and 8-Oxo-dG was quantitated by i-Solution software and shown as a bar graph. *, #, ** P<005 vs. normal kidney. (Panel F') Besides lupus nephritis, 9 other case of nephritis were stained with an Nrf2 antibody as control, and representative images were shown (Magnification ×400).

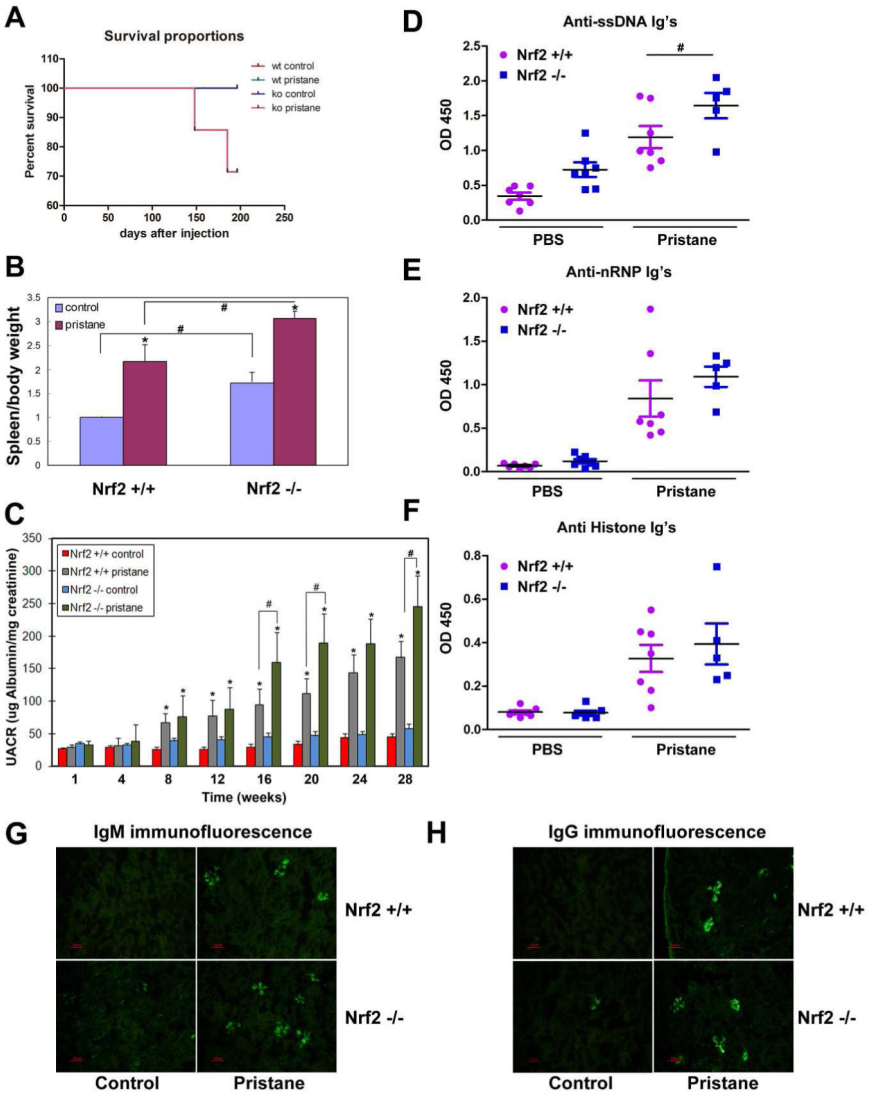

Figure 2. Renal damage induced by pristane treatment is more severe in Nrf2$^{-/-}$ mice

During the course of 28-week post-pristane injection, the survival of mice was monitored (2A), and the UACR was measured (2C). At the time of sacrifice, the body weight and spleen weight were measured and the ratio was calculated (2B). ELISA kits were used to measure the total Igs of anti-double strand DNA (2D), anti-nRNP (2E) and anti-histone (2F) in sera (1:50 dilution). Using anti-IgM and anti-IgG antibodies, the immunofluorescence was performed to measure the deposition within glomeruli (2G, 2H). *p<0.05, control *vs.* pristane treated; $^{\#}$p<0.05, Nrf2$^{+/+}$ *vs.* Nrf2$^{-/-}$.

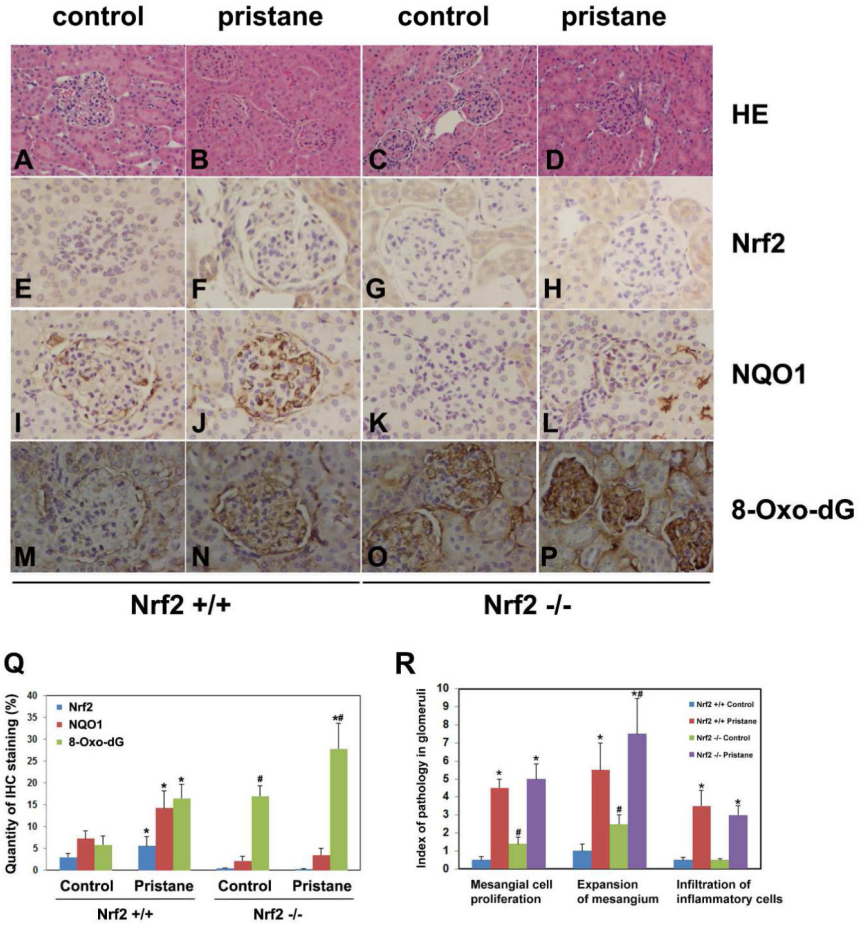

Figure 3. Oxidative DNA damage and elevated Nrf2 expression are observed in the glomeruli of lupus nephritis mice

The kidneys from Nrf2$^{+/+}$ and Nrf2$^{-/-}$ mice were isolated at the time of 28-week post-pristane injection. Pathology and IHC analysis were performed. (Panel A-D) Pathological diagnosis of renal injury. (Panel E to L) IHC staining with an anti-Nrf2 or anti-NQO1 antibody. (Panel M to P) IHC staining with an anti-8-Oxo-dG antibody. The staining of Nrf2, NQO1 and 8-Oxo-dG was quantitated by i-Solution software, and shown as a bar graph (3Q). * P<0.05 vs. control; # P<0.05 Nrf2$^{-/-}$ vs. Nrf2 $^{+/+}$. The pathological changes were scored and shown as a bar graph (3R). * P<0.05 vs. control; # P<0.05 Nrf2$^{-/-}$ vs. Nrf2 $^{+/+}$.

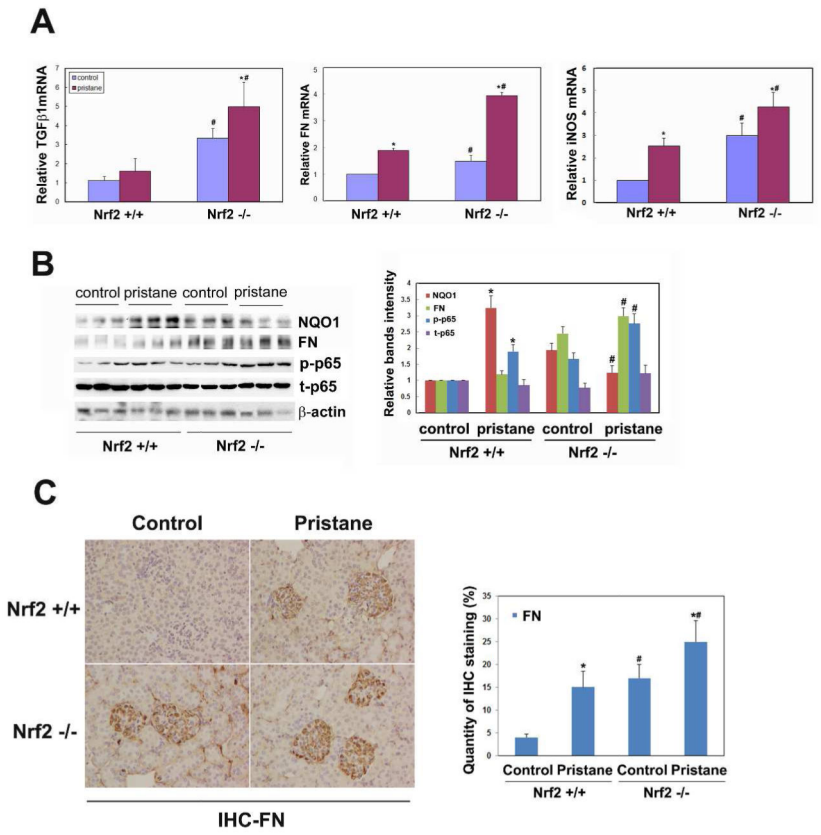

Figure 4. Increased expression of TGFβ1, fibronectin and iNOS in Nrf2$^{-/-}$ mice, compared to Nrf2$^{+/+}$ mice

(4A) The mRNA level of TGF-β1, FN and iNOS was measured by real-time RT-PCR. The data presented are relative mRNA levels normalized to β-actin, and the value from the Nrf2$^{+/+}$ control group was set as 1. *p<0.05, control *vs.* pristane treated; $^{\#}$p<0.05, Nrf2$^{+/+}$ *vs.* Nrf2$^{-/-}$, N=7. (4B) The protein level of NQO1, fibronectin, β-actin, phospho-p65 and total p65 was measured by immunoblot analysis. The intensity of each individual band was measured and normalized to its β-actin. * P<0.05 vs. control; # P<0.05 Nrf2$^{-/-}$ vs. Nrf2 $^{+/+}$. (4C) IHC staining with an anti-fibronectin antibody, the staining were quantitated by i-Solution and showed in bar graph. * P<0.05 vs. control; # P<0.05 Nrf2$^{-/-}$ vs. Nrf2 $^{+/+}$.

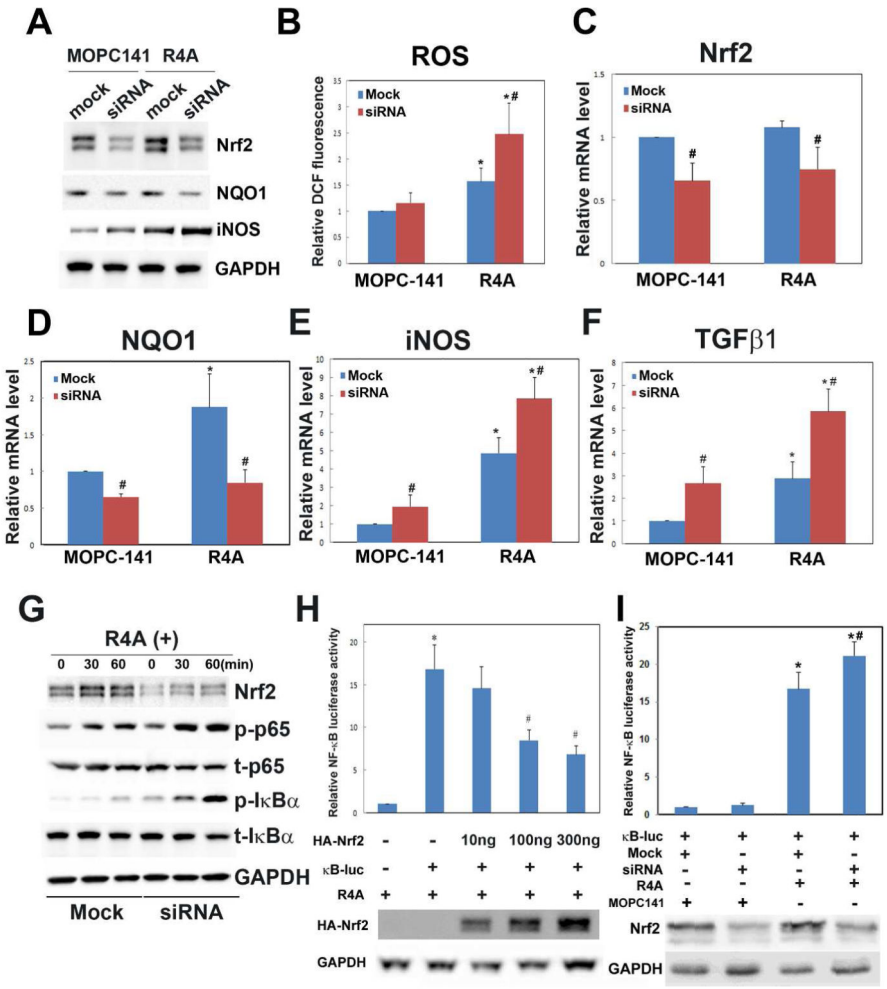

Figure 5. Nrf2 negatively regulates R4A-induced iNOS expression by suppressing the NF-κB signal pathway

Primary mesangial cells were isolated from as described in the method section. Following transfection with control-siRNA or Nrf2-siRNA for 24 h, mesangial cells were starved for 12-16 h before treated with R4A or MOPC-141. (5A) Immunoblot analysis with antibodies against Nrf2, NQO1, iNOS, or GAPDH in cells treated with an R4A or MOPC-141 antibody for 12 h. (5B) Cells were first incubated with DCF for 30 min, then treated for 2 h with either R4A or MOPC-141. The level of ROS was analyzed by flow cytometry. (5C-F) Cells were treated with R4A or MOPC-141 for 12 h. The mRNA level of Nrf2, NQO1, iNOS, and TGFβ1 was measured by real-time RT-PCR. (G) Cells treated with R4A or MOPC-141 for 0, 30 and 60 min were subjected to immunoblot analysis with an antibodies against Nrf2, phosphorylated p65, p65, phosphorylated IκBα, IκBα, and GAPDH. (H) Cells were transfected with plasmids for κB-firefly luciferase and TK-renilla luciferase (internal control), along with different amounts of an expression vector for Nrf2. At 24 h post-transfection, cells were treated with R4A for 12 h and then firefly and renilla luciferase activities were measured (upper panel). An aliquot of cell lysates was used for immunoblot analysis (lower panel). All the experiments were repeated three times and data represent means ± SD. (I) Cells were knockdown of Nrf2 by siRNA, then transfected with plasmids

for κB-firefly luciferase and TK-renilla luciferase. At 24 h post-transfection, cells were treated with R4A for 12 h and then firefly and renilla luciferase activities were measured (upper panel). An aliquot of cell lysates was used for immunoblot analysis (lower panel).

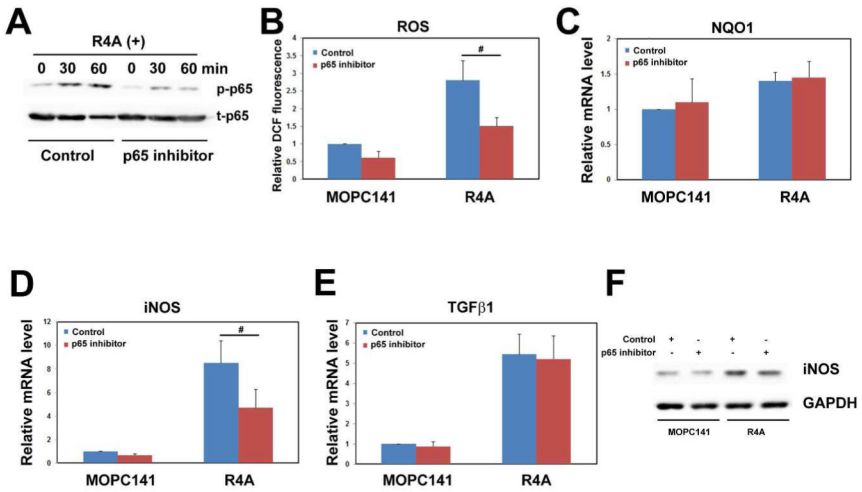

Figure 6. Inhibition of NF-κB pathway alleviated the production of ROS and expression of iNOS induced by R4A

The cells were incubated with an NF-κB p65 inhibitory peptide (50 μM) for 1 hour, then treated by R4A for indicated times. The cell extracts were subjected to immunoblot using anti-phospho-p65 and anti-total-p65 antibodies (6A). Cells were incubated with a p65 inhibitor (50 μM) for 1 hour and DCF for 30 minutes, and then treated with R4A for 2 hours. The level of ROS was analyzed by flow cytometry (6B). Cells were incubated with a p65 inhibitor (50 μM) for 1 hour, and then treated with R4A for 12 hours. The mRNA level of NQO1, iNOS, and TGFβ1 was measured by real-time RT-PCR (6C-E). The protein level of iNOS was measured by immunoblot (6F).

Table 1

Selected renal biopsies from lupus nephritis patients

Classification	Case (N)	Patients
Lupus nephritis		39
Class I	4	
Class II	9	
Class III	9	
Class IV	10	
Class V	7	
Normal kidney		12
Total		51