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Transcription factor Nrf2 is protective during ischemic and nephrotoxic acute kidney injury in mice

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Oxidative stress is involved in acute kidney injury due to ischemia–reperfusion and chemotherapy-induced nephrotoxicity. To investigate their basic mechanisms we studied the role of nuclear factor-erythroid 2-p45-related factor 2 (Nrf2), a redox-sensitive transcription factor that regulates expression of several antioxidant and cytoprotective genes. We compared the responses of Nrf2-knockout mice and their wild-type littermates in established mouse models of ischemia–reperfusion injury and cisplatin-induced nephrotoxicity. Several Nrf2-regulated genes encoding antioxidant enzymes/proteins were significantly upregulated in the kidneys of wild type but not Nrf2-knockout mice following renal ischemia. Renal function, histology, vascular permeability, and survival were each significantly worse in the Nrf2 knockout mice. Further, proinflammatory cytokine and chemokine expression tended to increase after ischemia in the knockout compared to the wild-type mice. Treatment of the knockout mice with the antioxidants *N*-acetyl-cysteine or glutathione improved renal function. The knockout mice were more susceptible to cisplatin-induced nephrotoxicity, and this was blunted by *N*-acetyl-cysteine pretreatment. Our study demonstrates that Nrf2-deficiency enhances susceptibility to both ischemic and nephrotoxic acute kidney injury, and identifies this transcription factor as a potential therapeutic target in these injuries.

Kidney International (2009) **76,** 277–285; doi:10.1038/ki.2009.157; published online 13 May 2009

KEYWORDS: acute kidney injury; antioxidant; cisplatin; ischemia-reperfuion; nephrotoxicity; transcription factor Nrf2

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Received 10 December 2007; revised 23 March 2009; accepted 1 April 2009; published online 13 May 2009

Ischemia–reperfusion injury (IRI) is a common cause of acute kidney injury (AKI) in both native and allograft kidneys. Ischemic AKI in native kidneys is associated with a high mortality rate of up to 50%.[1–3] Ischemic injury to renal allograft increases length of hospitalization and leads to increased short- and long-term allograft loss. Another common cause of AKI is nephrotoxicity, which limits the use of many effective therapeutic agents, including the chemotherapeutic agent cisplatin.[4] Elucidating the pathogenic mechanisms underlying AKI is critical to develop new diagnostic tools and therapeutic strategies.

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is a basic leucine zipper redox-sensitive transcription factor that regulates the expression of several cellular antioxidant and cytoprotective genes. Nrf2-mediated transcriptional responses have been shown to be protective in various experimental diseases including LPS-induced sepsis (shock),[5,6] oxidative lung injury and fibrosis, asthma, smoking-induced emphysema,[7–11] and brain IRI.[12,13] A recent study[14] has demonstrated elevated levels of Nrf2 and its downstream target genes in the kidney tissue of wild-type mice following induction of renal ischemia and reperfusion. However, the functional role of Nrf2 in AKI remains unclear. Because emerging evidence suggests that Nrf2 protects the lung and brain from acute injury through the regulation of antioxidant gene expression,[8] and oxidative stress is known to occur in AKI,[15] we hypothesized that Nrf2 deficiency would increase susceptibility to both ischemic and nephrotoxic AKI. We first performed gene expression profiling in the kidney of wild-type (Nrf2+/+) and Nrf2-knock out (Nrf2−/−) mice subjected to renal IRI. We found that several antioxidant pathways were deregulated during ischemic AKI in Nrf2−/− mice. Nrf2−/− mice had more pronounced changes in renal function, histology, proinflammatory protein expression as well as microvascular permeability than those seen in Nrf2+/+ mice with ischemic AKI. We reconstituted the antioxidant activity in the Nrf2−/− mice with *N*-acetyl cysteine (NAC) or glutathione (GSH) to partially mitigate the effects of Nrf2 deficiency. Using a cisplatin-induced AKI model, we found that Nrf2-deficiency also lowered the threshold for nephrotoxic injury and this was influenced by NAC.

RESULTS

Upregulation of Nrf2 target genes in the kidneys of mice subjected to ischemia–reperfusion

Using Affymetrix chips, our team previously demonstrated upregulation of Nrf2 target genes in the kidney of NIH-Swiss wild-type mice after moderate ischemia (30 min).[14] In current study, we explored whether Nrf2-regulated genes were altered in a different strain of mouse [Nrf2+/+ with CD1 background and the same mice with the Nrf2 gene deleted (Nrf2−/−)] after 60 min renal ischemia and used a less expensive array gene chip platform (Illumina, San Diego, CA, USA). At 24 h after renal IRI, four of 15 tested Nrf2 target genes were significantly upregulated in the kidney of Nrf2+/+ mice, but not in those of Nrf2−/− mice. All upregulated genes in the ischemic kidneys had a mean fold of change (FOC) of 1.5 or greater than those in the sham-operated kidneys and with a q-value less than 5%. These genes were aldehyde dehydrogenase (Aldh1a1, FOC: 1.91), growth arrest/DNA damage-inducible 45 (GADD45, FOC: 2.37), and glutathione S-transferase (Gsta2, FOC: 3.36; Gstp1, FOC: 1.89). (Figure 1).

Nrf2−/− mice had worse renal function than Nrf2+/+ mice after ischemia

We then evaluated the direct role of Nrf2-deficiency on renal function following renal IRI. Nrf2−/− and Nrf2+/+ mice underwent 30 min bilateral renal ischemia followed by reperfusion for up to 72 h. Serum creatinine (SCr) concentration was measured as a marker of renal function at 0 (baseline), 24, 48, and 72 h after ischemia. All animals survived to 24 h but four of six Nrf2−/− mice and two of five Nrf2+/+ mice died by 72 h post ischemia. After ischemic injury, Nrf2−/− mice had significantly increased SCr at 24 h (SCr in mg/dl, Nrf2−/− vs Nrf2+/+: 2.67 ± 0.21 vs 1.50 ± 0.29, $P<0.03$) and a trend towards an increase at 48 and 72 h when compared with wild-type mice (Figure 2a).

Nrf2−/− mice had increased renal vascular permeability after ischemia

We examined the effect of Nrf2 on renal microvascular injury by using extravasation of albumin-bound Evans blue dye as a marker of renal vascular permeability. Mice were subjected to either 30 min of renal ischemia followed by 24 h reperfusion or sham surgery. We found that after IRI, both Nrf2+/+ mice and Nrf2−/− mice had significantly greater renal Evans blue dye extravasations compared with the sham mice. However, the Evans blue dye extravasations after IRI was elevated in the kidneys of Nrf2−/− mice compared with Nrf2+/+ mice. (Evans blue dye extravasations in μg/g: 797 ± 71 vs 605 ± 36. $P<0.03$, Figure 2b).

Nrf2−/− mice had increased renal tubular injury after ischemia

Kidneys from both Nrf2−/− and Nrf2+/+ mice were harvested at 72 h post ischemia and processed for histological examination with hematoxylin and eosin stain. A renal pathologist (MH), blinded to the experimental groups, scored tubular injury semiquantitatively, by focusing on epithelial necrosis and infiltration of polymorphonuclear leukocytes (PMN). Nrf2−/− mice had greater tubular injury (Score: 3.71 ± 0.18 vs 3.14 ± 0.14, $P<0.03$; $n=7$) and PMN infiltration (score: 2.57 ± 0.30 vs 2.01 ± 0.29, $P<0.03$; $n=7$) when compared with Nrf2+/+ mice (Figures 2c and 3).

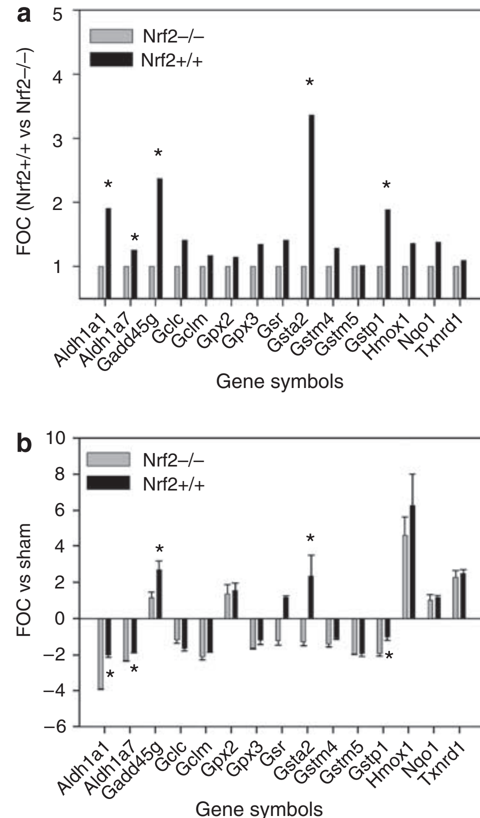

Figure 1 | Up/downregulated Nrf2 antioxidant genes in the ischemic kidney. Nrf2+/+ (wild type) and Nrf2−/− (deficient) mice underwent renal ischemia followed by reperfusion for 24 h. Total RNA was isolated from the collected kidneys and processed for gene microarray. The data is expressed as a fold change of each gene in ischemic kidney from wild-type mice over that from Nrf2−/− mice (**a**) or each gene in ischemic kidney over that in sham-operated kidney (**b**). (*$P<0.05$ vs Nrf2−/−). Gene symbols: Aldh1a1 (7), aldehyde dehydrogenase family 1, subfamily A1 (7); Gadd45, growth arrest/DNA-damage-inducible 45; Gclc (m), glutamate-cysteine ligase, catalytic (modified) subunit; Gpx2(3), glutathione peroxidase 2 (3); Gsr, glutathione reductase 1; Gsta2, glutathione S-transferase, α-2; Gstm4(5), glutathione S-transferase, mu 4 (5); Gstp1, glutathione S-transferase, pi 1; Hmox1, heme oxygenase (decycling) 1; Nqo1, NAD(P)H dehydrogenase, quinone 1; Txnrd1, thioredoxin reductase 1.

Nrf2−/− mice had increased mortality rate after severe renal ischemia

Thirty-minute bilateral renal ischemia is a moderate injury model that has a relatively low mortality rate. To evaluate whether Nrf2 deficiency worsens AKI-induced mortality, we subjected Nrf2−/− mice and Nrf2+/+ mice to 60 min bilateral renal ischemia (severe ischemia model) and analyzed mortality rates over the next 72 h. All 17 Nrf2−/− mice died by 72 h post ischemia whereas only two of eight (2/8) Nrf2+/+ mice died by 72 h, demonstrating a survival

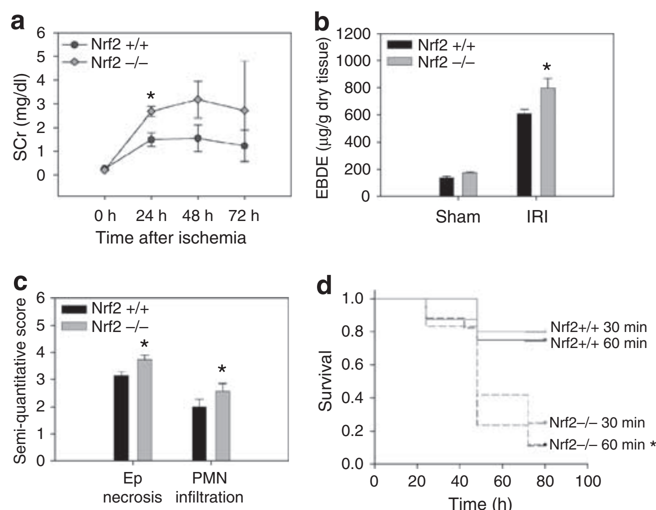

Figure 2 | Comparison of renal function, kidney histology, kidney vascular permeability, and survival in Nrf2 + / + and Nrf2−/− mice after renal ischemia. All mice underwent 30 (**a–d**) or 60 (**d**) min bilateral renal ischemia followed by reperfusion for up to 72 h. Serum creatinine (SCr), Evans blue dye extravasations (EBDE), tubular injury score and mouse survival was analyzed. (**a**: SCr. $^*P<0.02$ vs Nrf2 + / + , $n=6$; **b**: EBDE. $^*P<0.03$ vs Nrf2 + / + , $n=6$–8; **c**: tubular injury score. $^*P<0.03$ vs Nrf2 + / + , $n=7$; **d**: survival. $^*P=0.008$ vs Nrf2 + / + 60 min by log-rank test. $n=7$–17 in 60 min ischemia; $n=5$–6 in 30 min).

disadvantage in the Nrf2−/− mice compared with Nrf2 + / + mice ($P=0.008$ by log-rank test; Figure 2d). In contrast, in mice that underwent 30 min renal ischemia, four out of six Nrf2−/− mice and two out of five Nrf2 + / + mice died by 72 h – the survival rate between these two genotypes was not significantly different (mean survival time: Nrf2−/−, 54 h vs Nrf2 + / + , 67 h, $P=0.15$; Figure 2d).

Proinflammatory mediators in ischemic kidney

To examine potential inflammatory mechanisms by which ischemic AKI was accentuated in Nrf2−/− mice, we measured proinflammatory mediator cytokine protein using Bio-Plex cytokine/chemokine assay techniques (similar to multiple ELISA combined) in the kidneys from both Nrf2 + / + mice and Nrf2−/− mice subjected to IRI or sham-surgery. The expression level of each cytokine/chemokine is presented as fold of change (FOC) of each protein in ischemic kidneys over the level of the same cytokine/chemokine in sham kidneys. At 72 h after ischemia, Nrf2−/− mice had a trend towards increase in measured cytokines and chemokines in ischemic kidney when compared with Nrf2 + / + mice. These proteins are IL-2 (Mean ± s.d.: 7.15 ± 1.88 vs 5.18 ± 1.78, $P=0.26$), IL-6 (Median: 2.33 vs 2.13, $P=0.82$), KC (Median: 14.1 vs 5.9, $P=0.05$) and G-CSF (Mean ± s.d.: 11.66 ± 8.33 vs 5.31 ± 2.79, $P=0.06$) and a trend towards a decrease in RANTES (Median: 2.45 vs 4.53, $P=0.29$, Figure 4).

Supplementation with antioxidants NAC or GSH improved AKI in Nrf2−/− mice

To test whether Nrf2 protects mice from renal IRI through an antioxidant pathway(s), we pretreated Nrf2−/− mice and

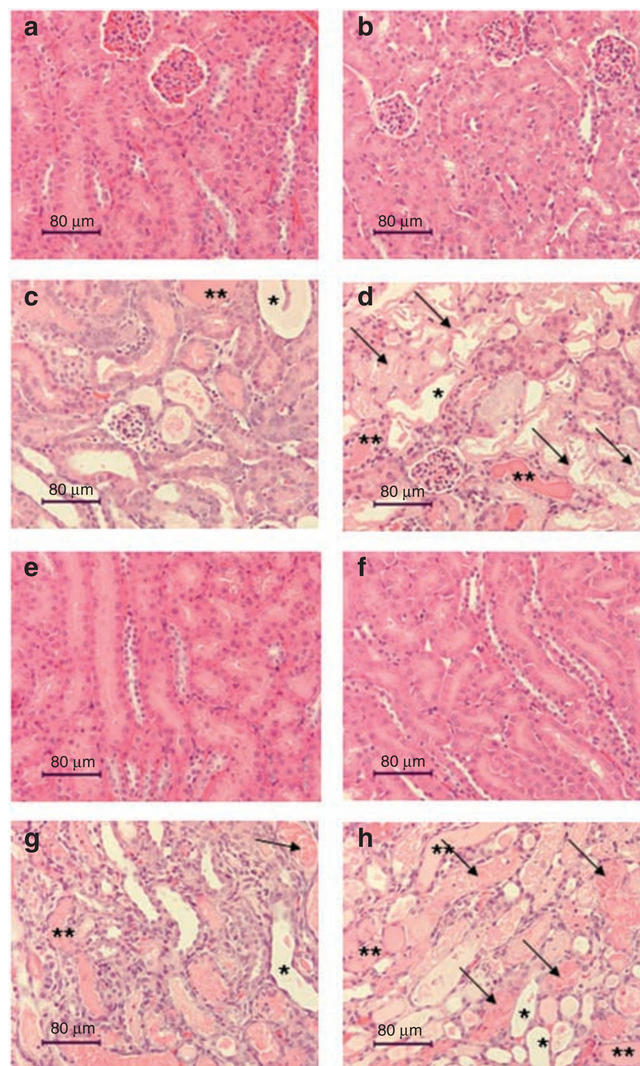

Figure 3 | Photomicrographs of kidney histology from Nrf2 + / + mice and Nrf2−/− mice. All mice underwent either 30 min bilateral renal ischemia or sham operation and were killed 72 h later. The collected kidneys were stained with H&E for histological examination. Nrf2−/− mice had more tubular injury than Nrf2 + / + mice after ischemia. (**a** and **e**: sham kidney from Nrf2 + / + mice; **b** and **f**: sham kidney from Nrf2−/− mice; **c** and **g**: ischemic kidney from Nrf2 + / + mice; **d** and **h**: ischemic kidney from Nrf2−/− mice. Asterisks indicate dilated tubules or cast formation. Arrows, necrotized tubules).

Nrf2 + / + mice with antioxidant NAC or GSH prior to ischemia. Control mice were treated with saline. All mice underwent 30 min bilateral renal ischemia and all mice were alive at 24 h after ischemia. Nrf2−/− mice treated with GSH or NAC had significantly smaller increases in serum creatinine level at 24 h than those pretreated with saline (SCr saline vs GSH vs NAC: 3.13 ± 0.13 vs 1.02 ± 0.45 vs 1.40 ± 0.37, $P<0.001$ by one-way ANOVA, $n=6$ in NAC or GSH and 13 in saline) (Figure 5a). In contrast, pretreatment of wild-type Nrf2 + / + mice with GSH or NAC did not improve renal function at 24 h after ischemia. (SCr: 2.71 ± 0.19 vs 2.52 ± 0.62 vs 2.53 ± 0.18, $P=0.87$, $n=4$–11) (Figure 5b). Histological analysis showed that pretreatment

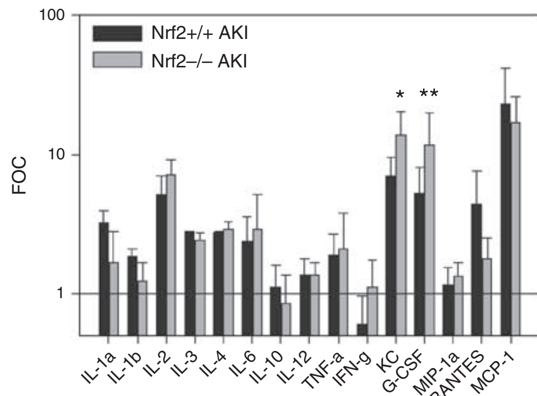

Figure 4 | Cytokine and chemokine proteins in the kidney. All mice underwent 30 min renal ischemia or sham operation and were killed at 72 h after surgery. Total protein was extracted from the kidneys and measured for cytokine and chemokine using Bio-plex protein array. The data are presented as fold of change (FOC) of each cytokine or chemokine in ischemic kidney over that in sham-operated kidney on a semi-log scale. (* $P=0.05$; ** $P=0.06$ vs Nrf2 +/+, $n=6$–8 per group).

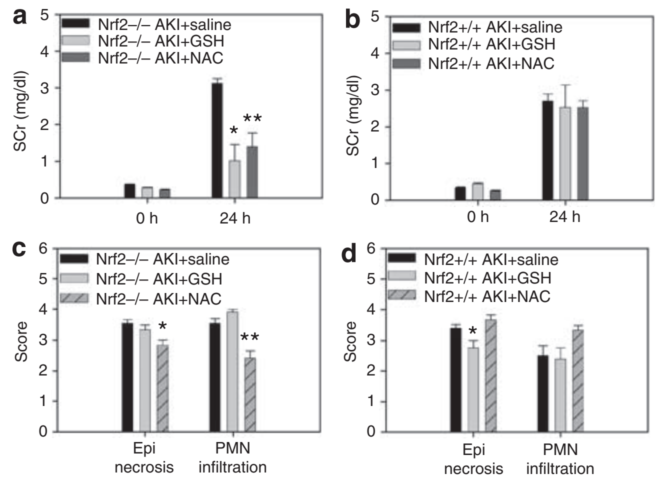

Figure 5 | Effect of GSH or NAC treatment on renal function and tubular injury score after ischemia. All mice were pretreated with either GSH (5 mM/kg/d i.p.) for 3 days, or NAC (500 mg/kg i.p.) 24 h or equal volume of saline before renal ischemia. Mice were killed at 24 h after surgery and serum creatinine concentration (**a** and **b**) was measured as the marker of renal function and kidney tubular injury score (**c** and **d**) was evaluated. **a**: SCr in Nrf2−/− mice. * $P<0.001$ and ** $P<0.003$ vs saline, $n=6$–13; **c**: * $P=0.011$ and ** $P=0.003$ vs saline, $n=6$–12; **d**: * $P=0.021$ vs saline, $n=3$–10. Epi: epithelium; PMN: polymorphonuclear leukocyte.

with NAC, but not GSH, improved epithelial necrosis (Median score: 3.5 vs 3.0, $P=0.011$ by Rank Sum test) and PMN infiltration (Median score: 4.0 vs 2.0, $P=0.003$, $n=6$–12) in Nrf2−/− mice after ischemia (Figure 5c). In Nrf2 +/+ mice, pretreatment with GSH improved tubule necrosis after ischemia (Mean ± s.e.m.: saline vs GSH vs NAC, 3.4 ± 0.13 vs 2.75 ± 0.25 vs 3.67 ± 0.17, $P=0.021$ by one-way ANOVA, $n=3$–10, Figure 5d).

Supplementation of NAC reduced proinflammatory mediators in ischemic kidney

We determined if the antioxidant pretreatment that improved renal function after IRI in Nrf2−/− mice could also decrease the generation of proinflammatory mediators in the ischemic kidney. We measured the protein levels of proinflammatory mediators by Bio-plex protein array in the ischemic kidneys from NAC pretreated Nrf2−/− mice and Nrf2 +/+ mice at 24 h after ischemia. We found that in both Nrf2−/− and Nrf2 +/+ mice, pretreatment with NAC mitigated the rise in IL-6 that occurred in mice pretreated with saline. (IL-6, median, saline vs NAC: Nrf2−/−, 12.7 vs 1.91, $P=0.004$; Nrf2 +/+, 50.2 vs 2.2, $P=0.04$; Figure 6).

Worse renal function and survival after cisplatin in Nrf2−/− mice compared with Nrf2 +/+ mice

We tested the effect of Nrf2 deficiency in mice in another clinical form of AKI, cisplatin-induced nephrotoxicity. All mice received a single intraperitoneal injection of cisplatin at a dose of 20 mg/kg and were observed for 7 days. During the follow-up, all four Nrf2−/− mice died between days 3 and 7, whereas only one of five Nrf2 +/+ mice died during this period. The survival rate of Nrf2 +/+ mice was higher than that of Nrf2−/− mice ($P<0.02$ by log-rank test; Figure 7b). The SCr in Nrf2−/− mice was significantly increased on day 3 to a level of 2.98 ± 1.12 mg/dl, whereas that of the

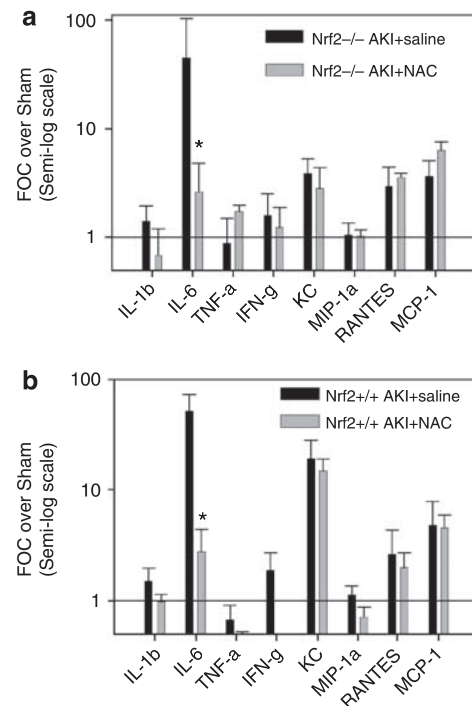

Figure 6 | Effect of NAC treatment on ischemic kidney cytokines and chemokines. All mice were pretreated with either NAC (500 mg/kg, i.p.) or saline 24 h before renal ischemia. The total protein was extracted from the harvested kidneys and processed for cytokine/chemokine protein array. **a**: Nrf2−/− mice, * $P=0.004$ vs saline; $n=8$–10. **b**: Nrf2 +/+ mice. * $P=0.04$ vs saline; $n=3$–8.

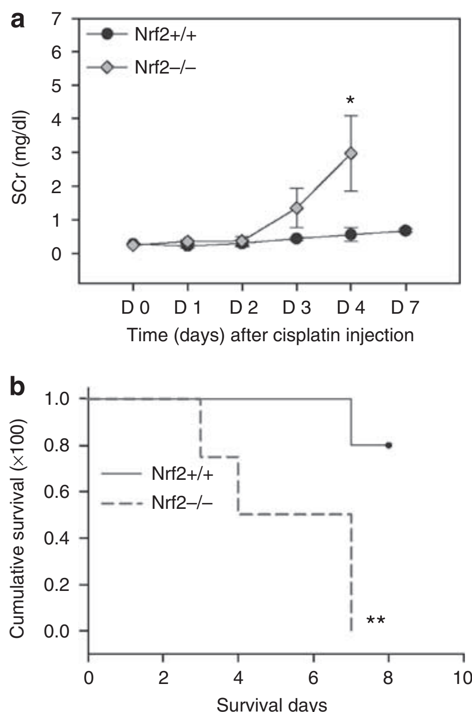

Figure 7 | Renal function and survival after lower dose of cisplatin. All mice were injected with cisplatin i.p. at 20 mg/kg and were followed up to 1 week. Serum creatinine concentration (**a**) was measured at an indicated time and the accumulative survival (**b**) was analyzed. (**a**: *$P<0.05$, $n=4$–5; **b**: **$P<0.02$ by log-rank test).

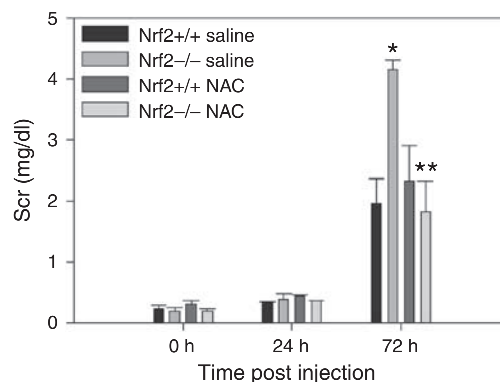

Figure 8 | Effect of NAC pretreatment on higher dose of cisplatin-induced renal dysfunction. All mice pretreated with either NAC (500 mg/kg i.p.) or saline 24 h before cisplatin injection (30 mg/kg, i.p.) and serum creatinine concentration was measured. (*$P<0.001$ vs Nrf2 +/+ saline; **$P=0.002$ vs Nrf2−/− saline; $n=5$ per group).

Nrf2 +/+ mice was only 0.47 ± 0.21 mg/dl ($P=0.049$, Figure 7a) at that time point.

Supplementation of antioxidant NAC reduced cisplatin nephrotoxicity in Nrf2−/− mice

To test if the enhanced susceptibility to cisplatin-induced nephrotoxicity in Nrf2-deficient mice was due to antioxidant mechanisms, we compared the effect of supplementation of NAC on renal function in mice with higher dose of cisplatin (30 mg/kg). We pretreated Nrf2−/− mice and Nrf2 +/+ mice with exogenous NAC (500 mg/kg, i.p.) 24 h before cisplatin injection. We found that Nrf2−/− mice with NAC, but not Nrf2 +/+ mice, had a significantly less increase in SCr at 24 h after cisplatin injection when compared with saline pretreated mice. (SCr: Nrf2−/−, saline vs NAC, 4.16 ± 0.14 vs 1.83 ± 0.49, $P<0.01$. Figure 8).

DISCUSSION

In this study, we demonstrated that genetic deficiency in Nrf2-mediated transcriptional responses, especially antioxidant pathways, enhances susceptibility to both ischemic and nephrotoxic AKI in mice. Our findings have revealed that after IRI, Nrf2-deficiency leads to increased mortality accompanied by augmented kidney dysfunction and vascular permeability. However, supplementation of exogenous antioxidants (NAC or GSH) to Nrf2−/− mice significantly improved renal function and histological injury, suggesting that oxidative stress may play a role in IRI-related kidney damage in the context of Nrf2-deficient redox signaling. Nrf2 deficiency also contributed to increased mortality and kidney dysfunction in a cisplatin-induced experimental model of AKI.

Gene expression profiling revealed that several antioxidant genes that are known to be regulated by Nrf2 were upregulated in ischemic kidneys of wild-type (Nrf2 +/+) mice, but not of Nrf2−/− mice. The upregulated genes included aldehyde dehydrogenase (ALDH1A1 and ALDH1A7), growth arrest and DNA damage (GADD45G), and glutathione *S*-transferases (GSTA2 and GSTP1). These results are consistent with those of a previous study that subjected a different strain of mice (NIH-Swiss) to 30 min renal ischemia and used the Affymetrix platform.[14] However, we did not find significant changes in the expression levels of the Nrf2-target gene, NADPH quinone oxidoreductase, in our current experimental conditions. Likewise, Leonard *et al.*[14] did not detect changes in the expression levels of GADD45G in their experimental conditions. It is unclear whether this discrepancy is due to genetic differences in mouse strain (NIH-Swiss vs CD-1 mice) or variations in experimental conditions, such as the different microarray platforms (Affymatrix vs Illumina) used in these two studies. Investigation that is more detailed is necessary to address this discrepancy.

Given that Nrf2-regulated genes were found to be upregulated in the post-ischemic kidney, we examined the role of Nrf2 by comparing renal IRI in Nrf2−/− mice and wild-type Nrf2 +/+ mice. We found that compared with wild-type mice, Nrf2−/− mice had significantly greater renal dysfunction as evidenced by a greater rise in serum creatinine at 24 h after ischemia, and greater histological injury with a higher tubular injury score, and increased renal vascular permeability following IRI. Thus, it appears that lower expression of Nrf2-related antioxidant genes contributes to increased kidney damage in Nrf2−/− mice during IRI. In support of this conclusion, a recent study

revealed that depressed expression of the Nrf2 gene might contribute to increased IRI in older donor liver after liver transplantation.[16]

To address whether Nrf2-regulated gene expression, especially the antioxidant response, confers protection against AKI from IRI, we supplemented Nrf2−/− mice with the antioxidants, NAC or GSH, prior to IRI. We found that pretreatment with either NAC or GSH significantly improved renal function (serum creatinine levels) after ischemia. However, we would like to point out that, although antioxidant supplementation significantly improved renal function, NAC or GSH supplementation only had a modest effect on kidney structural improvement in Nrf2-mice subjected to IRI. It is likely that Nrf2-regulated gene expression other than antioxidant responses may play a role in the repair of kidney structural injury caused by IRI. In support of this notion, we have shown by expression profiling that Nrf2 regulates gene expression in both a GSH-dependent and a GSH-independent manner in lung epithelial cells.[17] Some of these include various growth factors and growth factor receptors as well as structural genes. Thus, it is possible that antioxidant gene expression could not completely restore Nrf2-regulated gene expression in Nrf2−/− mice. Alternatively, it is possible that antioxidants can directly or indirectly regulate creatinine levels in Nrf2−/− mice, either at the level of creatinine generation, clearance or both.

Antioxidant (NAC) supplementation has been shown to be protective in number rodent models of experimental kidney IRI.[18–21] For example, de Araujo *et al.*[22] have shown that NAC supplementation confers protection against ischemic AKI, when combined with magnesium supplementation. However, Pincemail *et al.*[23] have shown that NAC dose not improve renal tubular injury in a rabbit model of IRI. We found that antioxidant supplementation had no significant effect on improving renal function in wild-type mice. Although we cannot rule out the possibility that Nrf2+/+ mice might be protected if treated at a different dose and or for a longer time, the protective effects of NAC on ischemia- and cisplatin-induced AKI in Nrf2-deficient mice, but not in wild-type mice suggests that both oxidative stress-dependent and independent mechanisms contribute to AKI. Alternatively, it is possible that antioxidant supplementation is only beneficial to the Nrf2−/− mice in the setting of a defective antioxidant response. It should be noted that antioxidant supplementation have yielded contradictory or inconclusive observations in several experimental models of AKI. In patients who underwent aortic/cardiac surgery or orthotropic heart transplantation, NAC treatment showed little renal protection.[24–26] Given the fact that Nrf2 is crucial for the induction of various networks of gene expression, including the antioxidant enzymes[27,28] and that NAC or GSH supplementation incompletely improves renal function in Nrf2−/− mice, it is very likely that Nrf2 regulates renal function and injury through other alternative pathways, in addition to GSH biosynthesis. For example, it has been shown that heme oxygenase, a transcriptional target of Nrf2, confers renal protection against ischemia-induced AKI.[29,30]

To further elucidate mechanisms by which Nrf2-deficiency enhances susceptibility to renal IRI, we examined several proinflammatory cytokine proteins in the kidneys from Nrf2+/+ mice and Nrf2−/− mice. We found trends toward higher levels of IL-6, KC, and G-CSF in Nrf2−/− mice compared with wild-type mice at 72 h after ischemia. KC is a putative biomarker as well as a mediator of AKI.[31] Therefore, an increase in the levels of KC is consistent with the functional and structural enhancement of damage in Nrf2-deficient mice. In contrast, Nrf2−/− mice displayed different levels of IL-1, IL-2, and IL-6 and diminished level of RANTES and MCP-1 at late time point as compared with wild-type mice. To determine whether the improvement in renal function afforded by exogenous antioxidant supplementation during ischemia–reperfusion was associated with attenuation of specific proinflammatory mediators in the kidney, we measured the levels of proinflammatory mediators in the ischemic kidneys from Nrf2−/− mice, and also Nrf2+/+ mice at 24 h after ischemia. We found that pretreatment with antioxidant reduced the increase in IL-6 levels both in Nrf2−/− mice and Nrf2+/+ mice when compared with vehicle-treated group, suggesting that Nrf2-dependent antioxidant response could lessen generation of proinflammatory mediators induced by ROS during IRI. Whether Nrf2 directly or indirectly modulates the expression levels of these cytokine/chemokines is unclear and warrants further study.

Nephrotoxicity is another leading cause of AKI.[4] To examine whether Nrf2 is also protective in nephrotoxic injury, we examined the effect of Nrf2 deficiency in a cisplatin-induced nephrotoxicity model. We found that Nrf2−/− mice had severe renal dysfunction and higher mortality than did Nrf2+/+ mice following administration of cisplatin. Similar to ischemic AKI, supplementation of NAC significantly improved renal function in cisplatin-treated Nrf2−/− mice. This finding has important implications for minimizing cisplatin toxicity by supplementation of exogenous antioxidant in patients who are deficient in endogenous antioxidant.

In summary, our data demonstrates that Nrf2 plays a protective role in experimental AKI, and that this protection is mediated, in part, through an endogenous antioxidant pathway. Modulating the Nrf2-regulated pathways may be an attractive target for future therapeutic studies in AKI, particularly in the setting of antioxidant deficient or other susceptible populations.

METHODS

Mice

CD-1 background Nrf2-deficient mice (*Nrf2–/–*) were generated as described earlier[27] and the wild-type mice (Nrf2+/+) with same background were used as controls. Breeding pairs of ICR/Sv129-*Nrf2*$^{+/-}$ mice were obtained from a colony at Tsukuba University (Tsukuba, Ibaraki, Japan) and maintained in the Johns Hopkins animal facility with air-conditioning and 12–10 h light–dark cycle

under a pathogen-free condition. All experimental protocols conducted in these mice were approved by the Johns Hopkins University Animal Care and Use Committee and were performed in accordance with NIH guidelines. All animals had free access to food and water during the experiments.

Renal ischemia–reperfusion injury model

An established model of renal IRI was used.[32] Animals were anesthetized with intraperitoneal injection of sodium pentobarbital at 75 mg/kg. Abdominal incisions were made and the renal pedicles were bluntly dissected. A microvascular clamp was placed on each renal pedicle for 30 or 60 min. During the procedure, animals were well hydrated with warm saline, (1 ml at the beginning of ischemia and 1 ml at the beginning of reperfusion) and mice body temperatures were maintained constantly at 35.5–37°C on a heating pad (40°C) until awake. After removing the clips, the wounds were sutured and the animals were allowed to recover.

Administration of cisplatin

Cisplatin (*cis*-diammineplatinum II dichloride, Sigma-Aldrich, St Louis, MO, USA) was dissolved in 0.9% saline at a concentration of 2 mg/ml. Mice were given a single i.p. injection either at a low dose of 20 mg/kg or at a higher dose of 30 mg/kg body weight. This high dose was chosen based on our preliminary studies that showed a lower dose did always give consistent and significant renal dysfunction. A dose at 30 mg/kg produced a predictable combination of survivability and acute renal failure as early as at 24 h and reached a peak at 72 h after cisplatin administration in C57BL6 wild-type mice in our previous report.[33]

Assessment of renal function

Blood samples were obtained from mice through tail vein prior to (0) and at 24, 48, and 72 h or day 4 and 7 after ischemia or cisplatin. Serum creatinine concentration was measured as a marker of renal function by a Roche Cobas Fara automated system (Roche, Nutley, New Jersey, USA) using a Creatinine 557 kit (Sigma Diagnostics, St Louis, Missouri, USA).

Evaluation of kidney histology

Formalin-fixed and paraffin-embedded kidney tissues were cut and stained with hematoxylin and eosin. Only the right kidney from each mouse was examined and scored. Renal tubular injury was assessed using a semi-quantitative scale. A renal pathologist (MH) who was blinded to the experimental groups scored the magnitude of tubular epithelial cell necrosis and interstitial PMN infiltration into five levels based on the estimation of the fraction of tubules with epithelial necrosis in the outer medulla and region of the cortico–medullary junction in a high power field under light microscope. We scored tubular injury according to the following schema (0: none; 0.5: $<10\%$; 1: 10–25%; 2: 25–50%; 3: 50–75%; 4: $>75\%$).[34] We also counted the number of neutrophils per high power ($\times 400$) field in 10 consecutive high power fields in the region of the cortico–medullary junction, starting with the field containing the greatest number of neutrophils. A neutrophil infiltration score was then determined according to the following scale[34]: 0, 0–1; 1, 2–10; 2, 11–20; 3, 21–40; 4, >40 or too many to count.

Renal vascular permeability

Renal microvascular injury secondary to ischemia was assessed by extravasation of albumin-bound Evans blue dye from the kidney parenchyma.[35] Thirty minutes before the mice were killed, Evans blue dye (Sigma Chemical Co., St Louis, MO, USA) (2 ml/kg 1% dye in 0.9% of saline) was injected through the right jugular vein whereas the animals received light ether anesthesia. Immediately after the animals were killed (by an overdose of pentobarbital), the circulation was cleared by transcardiac perfusion using 10 ml of heparinized saline (0.5 ml of heparin in 500 ml of 0.9% NaCl). The flushed kidneys were removed and dried at 60°C for 3 days. The dried kidneys were weighed and homogenized in formamide with a ratio of 1–20 (w/v). The homogenates were incubated at 60°C for 18 h (overnight), and then centrifuged for 30 min at 14,000 r.p.m. at 4°C to remove suspended particulate matter. The quantity of extracted EB in the supernatant was determined by measuring absorbance at a dual-wavelength of 620/635 nm and corrected for the extraction volume. A standard curve of Evans blue in a blank formamide was used to convert absorbency into micrograms of Evans blue per gram of dried tissue.

Gene microarray in the kidney

Total RNA was extracted from kidney tissues using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA 92008, cat. no. 15596-026). Additional purification was performed on RNAeasy columns (Qiagen, Valencia, CA, USA 913555, cat. no. 74104). The quality of total RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples were labeled according to Illumina recommended protocols. In brief, 0.5 μg of total RNA from each sample was labeled by using the Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX, USA 78744-1832, cat. no. IL1791) in a process of cDNA synthesis and *in vitro* transcription similar to that of other chip manufacturers used. Single-stranded RNA (cRNA) was generated and labeled by incorporating biotin-16-UTP (Roche Diagnosics GmbH, Mannheim, Germany, cat. no. 11388908910). 0.85 μg of biotin-labeled cRNA was hybridized (16 h) to Illumina's Sentrix MouseRef-8 Expression BeadChips (Illumina 92121-1975). The hybridized biotinylated cRNA was detected with streptavidin-Cy3 and quantitated using Illumina's BeadStation 500GX Genetic Analysis Systems scanner.

Analysis of array data

Preliminary analysis of the scanned data was performed using Illumina BeadStudio software. The basic Illumina data is returned from the scanner in the form of an '.idat' file, which contains single intensity data values/gene following the computation of a trimmed mean average for each probe type represented by a variable number of bead probes/gene on the array. The Bead Studio software returns information on the number and s.d. of all the bead measurements per probe/gene. A detection call based on a comparison between the measured intensity for a single probe/gene, and the intensities measured for a large number of negative control beads built-in to the BeadChip arrays is also given. ($D=$%above negative/100, $1=$perfect, that is, the intensity value of a gene is greater than all the intensities for every negative control tested) Background filtering, defined as any gene below $D=0.95$ for 75% of samples, was eliminated from further analysis. Then the data was log transformed and normalized to chipwise mean using CLUSTER tool[36] and processed using Significance Analysis of Microarray (SAM) software. Fold changes and false discovery rate were computed by Significance Analysis of Micro arrays (SAM) method[37] using 1000 permutations and 1.5-fold change as cutoff values. Gene with false discovery rate ($Q<5\%$) were considered significantly affected by IRI and dynamically link to Gene Ontology (GO) terms using tabulated data Mouse430A_2 Annotations, CSV (8.6 MB, 4/14/06) obtained

from NetAffx provided databases (http://www.affymetrix.com/support/technical/byproduct.affx?product=moe430A-20). As the mouse GO is not yet complete, we enriched mouse GO with orthologous human GO terms using Mouse430A_2 Annotations, Orthologs (4.6 MB, 4/14/06). The mouse/human GO database was then linked to IRI-related candidate genes and searched for endothelium and cell adhesion terms.

Cytokines/chemokines protein array

To examine proinflammatory molecules generated by ischemia–reperfusion injury, protein levels of IL-1α/β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IFN-γ, TNF-α, KC, G-CSF, MIP-1α, RANTES, and MCP-1 were measured in mice kidneys by Bio-Plex multiple cytokine array (Bio-Rad Laboratories Inc., Hercules, CA, USA) earlier described in depth.[38] Briefly, a portion of snap frozen tissue was homogenized in a cell-lysis buffer, and the homogenates were centrifuged at 12,000 r.p.m. for 15 min at 4°C. Total protein concentration in each supernatant was determined using a Bio-Rad Protein Assay Kit, and the measured protein level in each sample was adjusted to 500 μg/ml with cell lysis buffer. Each sample was first incubated with a mixture of all types of micro-beads for 90 min at room temperature followed by incubation with biotinylated detection antibodies for 30 min, then with a streptavidin-coupled phycoerythrin for 10 min room temperature. Finally, the samples were subjected to a flow cytometric system. All acquired data was analyzed using Bio-Plex Manager 3.0 software (Bio-Rad).

Administration of *N*-acetyl-cysteine or gluthathione

To examine the effect of replenishing antioxidant in Nrf2 deficiency on renal IRI, a group of Nrf2−/− mice was pretreated either with *N*-acetyl cysteine (NAC, Sigma-Aldrich) at 500 mg/kg body weight (i.p.) 24 h and 1 h before ischemia. The dose and timing of NAC administration were based on and modified from the study which showed NAC improved survivals in both of Nrf2−/− mice and wild-type mice from lethal dose of LPS.[5] Another group of mice were pretreated with glutathione-reduced ethyl ester (GSH, Sigma-Aldrich) at 5 mM/kg (i.p.) per day for 3 days before ischemia. This dose and timing was chosen based on our previous studies.[39,40]

Statistics

Statistical analysis was performed with professional statistics software SigmaStat (3.1) and all quantitative or semi-quantitative data presented in this study were automatically checked for assumption before performing any of the comparisons. Data passed both Normality and Equal Variance tests are presented as mean ± s.e.m. and the data failed either of assumption tests are presented as median with confidence. Single comparison between two groups was performed with Mann–Whitney rank sum *t*-test whether assumption tests failed or with an unpaired, two-tailed Student's *t*-test whether assumption tests passed. Multi-comparison among three or more groups was performed by an ANOVA *post hoc* test. Cumulative survival was analyzed by Kaplan–Meier log-rank test. Statistical significance of difference was defined when the P-value <0.05.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This study was supported by grants from NHLBI PO HL073944 (HR & SPR), NIH 2R01 DK54770 (HR) and grants from the National Kidney Foundation (DNG & ML).

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