The American Journal of Pathology, Vol. 189, No. 6, June 2019

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The American Journal of
PATHOLOGY
ajp.amjpathol.org

CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Loss of the Na^+/H^+ Exchange Regulatory Factor 1 Increases Susceptibility to Cisplatin-Induced Acute Kidney Injury

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Accepted for publication February 25, 2019.

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Na^+/H^+ exchange regulatory cofactor (NHERF)-1, a scaffolding protein, anchors multiple membrane proteins in renal proximal tubules. Cultured proximal tubule cells deficient in Nherf1 and proximal tubules from Nherf1-deficient mice exhibit aberrant trafficking. Nherf1-deficient cells also exhibit an altered transcription pattern and worse survival. These observations suggest that NHERF1 loss increases susceptibility to acute kidney injury (AKI). Male and female wild-type C57BL/6J and *Nherf1* knockout mice were treated with saline or cisplatin (20 mg/kg dose i.p.) to induce AKI and were euthanized after 72 hours. Blood and urine were collected for assessments of blood urea nitrogen and neutrophil gelatinase–associated lipocalin, respectively. Kidneys were harvested for histology (hematoxylin and eosin, periodic acid-Schiff) and terminal deoxynucleotidyl transferase dUTP nick end labeling assay, *Kim1* mRNA assessment, and Western blot analysis for cleaved caspase 3. Cisplatin treatment was associated with significantly greater severity of AKI in knockout compared with wild-type mice, as demonstrated by semiquantitative injury score (2.8 versus 1.89, $P < 0.001$), blood urea nitrogen (151.8 ± 17.2 mg/dL versus 97.8 ± 10.1 mg/dL, $P < 0.05$), and neutrophil gelatinase–associated lipocalin urine protein (55.6 ± 21.3 μg/mL versus 2.7 ± 0.53 μg/mL, $P < 0.05$). Apoptosis markers were significantly increased in cisplatin-treated *Nherf1* knockout and wild-type mice compared to respective controls. These data suggest that NHERF1 loss increases susceptibility to AKI. *(Am J Pathol 2019, 189: 1190–1200; https://doi.org/10.1016/j.ajpath.2019.02.010)*

Cisplatin is a chemotherapeutic agent widely used in treating a variety of solid malignant tumors (eg, ovarian, testicular, head and neck, and lung cancers).[1,2] Cisplatin was approved by the US Food and Drug Administration in 1978 for the treatment of advanced ovarian and bladder cancers and is one of the most widely used antitumor drugs in the world. Unfortunately, the major dose-limiting factor of cisplatin is its associated nephrotoxicity. Cisplatin-related nephrotoxicity can present in a variety of ways, but the most serious and common presentation is acute kidney injury (AKI). AKI complicates 1% to 7% of all hospital admissions and 1% to 25% of intensive care unit admissions,[1,3,4] increases the risk for death by 10- to 15-fold, and is associated with a 50% mortality rate.[1,5,6] Currently, 20% to 30% of patients receiving a single dose of cisplatin will develop AKI.[7] Clinically, cisplatin-related nephrotoxicity typically develops after 10 days of administration, and the prevalence of nephrotoxicity increases with repeated doses.[8] Permanent nephrotoxicity can occur if successive treatment courses are

Supported in part by US Department of Veterans Affairs Biomedical Laboratory Research and Development Service Merit Review Award R01DK083361 (E.D.L.).

Disclosures: E.D.L. is a consultant for WebMD and Healthcare Quality Strategies, Inc. (HQSI).

The views in the manuscript are those of the author and do not reflect the views of the US Department of Veterans Affairs.

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https://doi.org/10.1016/j.ajpath.2019.02.010

continued.[2] In addition, cisplatin-induced AKI frequently progresses to chronic kidney disease, a risk factor for early death. Despite extensive research, the mechanism of cisplatin-induced AKI is not completely understood, and there are currently no US Food and Drug Administration–approved therapies to halt or reverse AKI progression. Furthermore, clinicians have no good tools for predicting the likelihood of cisplatin-related nephrotoxicity in the individual patient. If a renoprotective therapy is to be developed, the mechanisms behind susceptibility to cisplatin-induced AKI and at-risk populations must be better understood and novel targets identified.

The goal of this study was to determine whether the expression of the scaffolding protein Na^+/H^+ exchange regulatory cofactor (NHERF)-1 influences the development of cisplatin-induced AKI. NHERF1 is a membrane-associated protein of the NHERF family of PDZ-scaffold proteins that contain two postsynaptic density protein-95/*Drosophila* discs large/zonula occludens 1 homology domains in the N-terminal half of the protein, and a C-terminal MERM (moesin, ezrin, radixin, merlin) domain (residues 242 to 358),[9,10] which anchors it to the cytoskeleton in the subapical plasma membrane region.[11] NHERF1 is expressed in all types of epithelial cells and provides a scaffold for multiprotein signaling complexes. In proximal tubule cells, NHERF1 is a key scaffolding protein for transport proteins and has a crucial role in defining the renal proximal tubule brush border membrane composition and in regulating ion transport.[9,11,12] Recent studies, however, suggest a much broader role for NHERF1 in human biology, including tumorigenesis,[13,14] generation of cellular reactive oxygen species,[15] and response to injury.[16] Studies have demonstrated that cholestatic liver injury induced by bile duct ligation is attenuated in *Nherf1* knockout (KO) mice, suggesting a role for NHERF1 in response to injury.[16] NHERF1 has also been implicated in renal injury. Spontaneously hypertensive rats and aging rats (22 months) show a marked decrease in renal Nherf1 expression.[17]

Previous unpublished observations from our laboratory comparing wild-type (WT) and Nherf1-deficient opossum kidney cells, a model of mammalian renal proximal tubule, demonstrated that although there were no apparent morphologic differences between the cell lines, the Nherf1-deficient cells grew more slowly and were more likely to die in culture. The Nherf1-deficient cells exhibited markedly diminished expression of multiple brush border membrane proteins, including the sodium phosphate cotransporter Npt2a.[18] Additionally, Nherf1-deficient cells in culture showed a decrease in the expression of *Npt2a* mRNA, which was rescued by transfection of Nherf1.[18] When transfected with Npt2a constructs, Nherf1-deficient cells showed faulty membrane protein trafficking.[18] These properties of the Nherf1-deficient cells (the poor survival, alterations in transcription pattern, and aberrant trafficking) suggest that NHERF1 deficiency may be associated with kidney injury. Based on these observations, we proposed the hypothesis that a loss of NHERF1 results in increased susceptibility to cisplatin-induced AKI.

Materials and Methods

Reagents and Antibodies

The following antibodies were purchased from Cell Signaling Technology (Beverly, MA): cleaved caspase 3 and glyceraldehyde-3-phosphate dehydrogenase. Horseradish peroxidase–conjugated secondary antibodies were purchased from Cell Signaling Technology. Pharmaceutical-grade cisplatin was purchased from the University of Louisville hospital pharmacy (Louisville, KY), and was used for animal experiments. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Animals and Treatments

Two-month–old male and female C57BL/6J WT and *Nherf1*$^{-/-}$ KO mice[19] were maintained on a 12-hour light–dark cycle and were provided water and food *ad libitum*. Mice received a single i.p. injection of 20 mg/kg cisplatin or vehicle (saline). Vehicle- and cisplatin-treated mice were euthanized after 72 hours. All studies were performed at the same time each day. Blood samples were collected from these mice and plasma was extracted via centrifugation (10,000 × *g* for 10 minutes) to measure blood urea nitrogen (BUN) levels. Kidneys were removed and decapsulated; one kidney was immersed in 3.7% formaldehyde in phosphate-buffered saline for 24 hours and transferred to 70% ethanol before paraffin embedding for histology, while the other kidney was decapsulated and collected in ice-cold phosphate-buffered saline with 1% penicillin/streptomycin. The cortex was separated from the medulla for homogenization, and portions of the kidney tissue were snap-frozen in liquid nitrogen for RNA isolation. All animals in these studies were used in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Louisville, and the guidelines of the American Veterinary Medical Association.

Histology and Semiquantitative Scoring

Whole kidney sections were embedded in paraffin and cut 5 μm thick either transversely or longitudinally from cisplatin- and vehicle-treated WT and *Nherf1* KO animals. These sections were stained with hematoxylin and eosin (H&E) as well as periodic acid-Schiff (PAS). A renal pathologist (S.C.) determined the degree of morphologic changes and injury in a blinded fashion. The following measures were chosen as an indication of morphologic damage to the kidney after treatment with vehicle or cisplatin: acute proximal tubular cell necrosis, loss of the brush border, proximal tubule cell detachment, and tubular casts. These measures were evaluated on a 4-point scale: 0 (none) indicates normal kidney; 1+ (mild) indicates primarily normal kidney with only occasional casts involving

<5% atrophy with spotty acute tubular necrosis; 2+ (moderate) indicates patchy injury with casts and atrophy involving 25% to 50% and spotty acute tubular necrosis; and 3+ (severe) indicates diffuse casts and atrophy >50% area along with spotty acute tubular necrosis. The numbers of mice per group were as follows: WT female, $n = 8$; *Nherf1* KO male, $n = 9$; WT male, $n = 11$; and *Nherf1* KO female $n = 11$.

Blood Urea Nitrogen and Neutrophil Gelatinase–Associated Lipocalin Measurements

BUN was determined from blood plasma using the Catalyst Dx Chemistry Analyzer and BUN Urea Test Clips (Idexx Laboratories, Westbrook, ME) for quantitative measurement. The numbers of mice per group were as follows: *Nherf1* KO male vehicle, $n = 5$; *Nherf1* KO female vehicle, $n = 5$; WT male vehicle, $n = 6$; WT male cisplatin, $n = 6$; *Nherf1* KO male cisplatin, $n = 6$; *Nherf1* KO female cisplatin, $n = 6$; WT female cisplatin, $n = 7$; and WT female vehicle, $n = 11$. Neutrophil gelatinase–associated lipocalin (NGAL) was determined using an enzyme-linked immunosorbent assay kit (catalog number DY1857; R&D Systems; Minneapolis, MN) on mouse urine, as directed by the manufacturer. The numbers of mice per group were as follows: *Nherf1* KO female vehicle, $n = 2$; *Nherf1* KO male vehicle, $n = 3$; WT male vehicle, $n = 3$; WT male cisplatin, $n = 4$; *Nherf1* KO male cisplatin, $n = 4$; *Nherf1* KO female cisplatin, $n = 5$; WT female vehicle, $n = 9$; and WT female cisplatin, $n = 11$.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from kidney cortex using the Ambion mirVana mRNA Isolation Kit (Thermo Fisher Scientific, Austin, TX) per the manufacturer's instructions. cDNA was synthesized from 1000 ng of total RNA using SuperScript Vilo MasterMix (Thermo Fisher Scientific) following the manufacturer's instructions. Kidney injury molecule (Kim)-1 (catalog number Mm00506686; Thermo Fisher Scientific) and the housekeeping gene β_2 microglobulin (*B2m*; catalog number Mm00437762; Thermo Fisher Scientific) were used in combination with TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Amplification reactions were performed using the Applied Biosystems 7500 Real-Time PCR System and Sequence Detection software version 1.3.1 (Applied Biosystems), with stage 1 (50°C for 2 minutes), stage 2 (95°C for 10 minutes), and 40 cycles of a 2-step PCR (95°C for 15 seconds, 60°C for 1 minute). Fluorescence intensity of each sample was measured at each cycle to monitor amplification of the target gene. The comparative cycle threshold method was used to determine fold-changes in mRNA expression compared to an endogenous reference gene (*B2m*). The numbers of mice per group were as follows: WT male vehicle, $n = 5$; WT female vehicle, $n = 5$; *Nherf1* KO male vehicle, $n = 5$; *Nherf1* KO female vehicle, $n = 5$; WT male

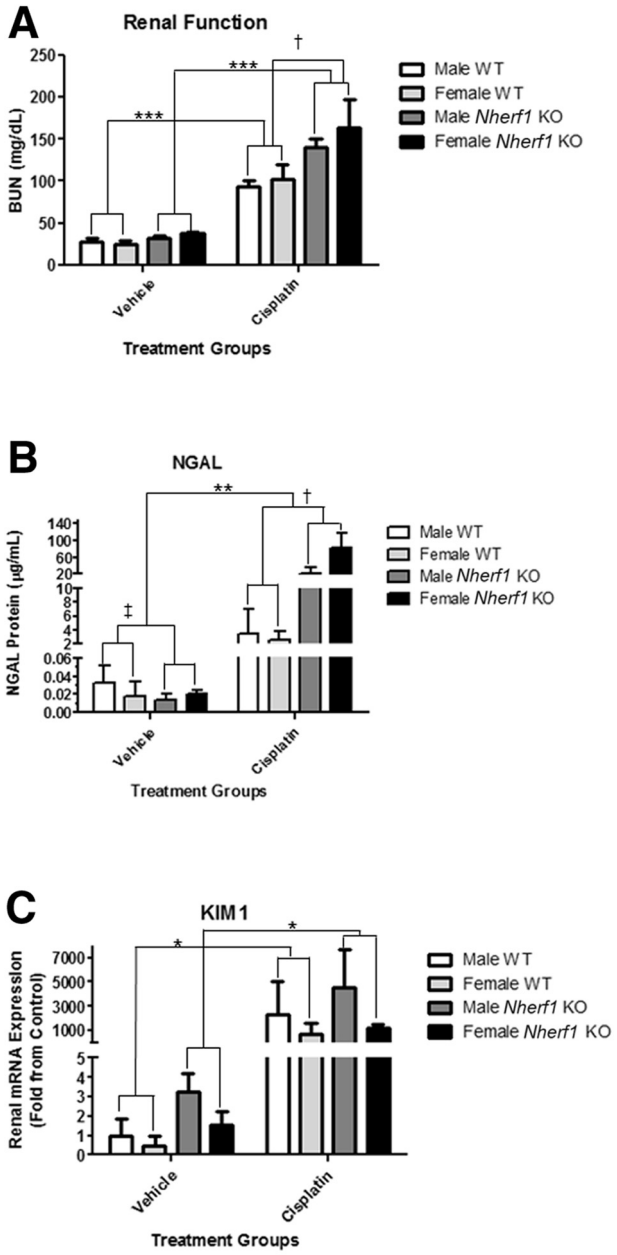

Figure 1 Effect of cisplatin on renal function in wild-type (WT) and *Nherf1* knockout (KO) mice. Two-month–old male and female C57BL/6J WT and *Nherf1* KO mice were given cisplatin (20 mg/kg dose i.p.) or vehicle (saline) and sacrificed after 72 hours as described in *Materials and Methods*. Renal function was determined. **A:** Blood urea nitrogen (BUN) measurement from mouse plasma. **B:** Neutrophil gelatinase-associated lipocalin (NGAL) protein measurement of mouse urine. **C:** Renal cortex mRNA expression of *Kim1* by quantitative RT-PCR. Data are expressed as means ± SEM. $n = 2$ (*Nherf1* KO female vehicle, **B**); $n = 3$ (WT male vehicle, *Nherf1* KO male vehicle, **B**); $n = 4$ (WT male cisplatin and *Nherf1* KO male cisplatin, **B**); $n = 5$ (*Nherf1* KO male vehicle and *Nherf1* KO female vehicle, **A**; *Nherf1* KO female cisplatin, **B**; WT male vehicle, WT female vehicle, *Nherf1* KO male vehicle, *Nherf1* KO female vehicle, WT male cisplatin, WT female cisplatin, *Nherf1* KO male cisplatin, and *Nherf1* KO female cisplatin, **C**); $n = 6$ (WT male vehicle, WT male cisplatin, *Nherf1* KO male cisplatin, and *Nherf1* KO female cisplatin, **A**); $n = 7$ (WT female cisplatin, **A**); $n = 9$ (WT female vehicle, **B**); $n = 11$ (WT female vehicle, **A**; WT female cisplatin, **B**). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.05$ (three-way analysis of variance).

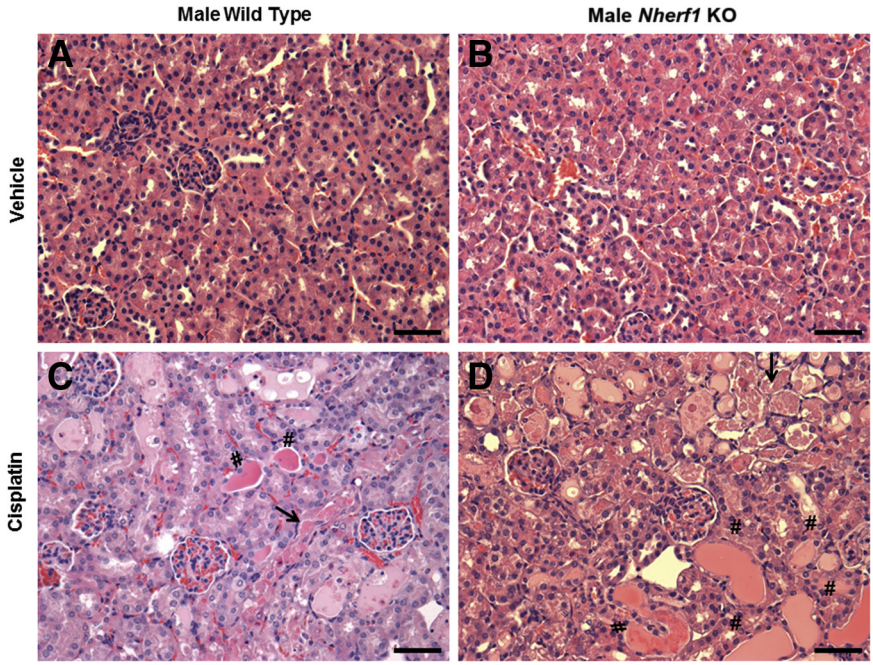

Figure 2 The histologic effect of cisplatin on hematoxylin and eosin (H&E) in male wild-type (WT) and *Nherf1* knockout (KO) mice. Representative photomicrographs of H&E staining of whole kidney sections embedded in paraffin and cut at 5 μm thick either transversely or longitudinally from cisplatin- and vehicle-treated WT and *Nherf1* KO male animals. **A**–**D:** A vehicle-treated male WT kidney (**A**), a vehicle-treated male *Nherf1* KO kidney (**B**), a cisplatin-treated male WT kidney (**C**), and a cisplatin-treated male *Nherf1* KO kidney (**D**). **Arrows** indicate acute tubular necrosis; **pound signs** indicate tubular casts. Scale bars: 50 μm. Original magnification, ×40.

cisplatin, $n = 5$; WT female cisplatin, $n = 5$; *Nherf1* KO male cisplatin, $n = 5$; and *Nherf1* KO female cisplatin, $n = 5$.

TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the ApopTag Plus Peroxidase *in situ* apoptosis kit (EMD Millipore, Billerica, MA) per the manufacturer's instructions. Whole kidney sections were counterstained with hematoxylin for automation in conjunction with 3,3-diaminobenzidine substrate to visualize cell nuclei. Quantitation of TUNEL-positive nuclei was conducted in a blinded fashion (A.B.-S.), and only positively stained nuclei exhibiting characteristics of apoptotic nuclei, such as nuclear condensation and fragmentation, were included. Furthermore, positively stained nuclei on the outer edges of the sections were omitted during analysis. Randomly chosen visual fields (20 to 30) were observed under a ×40 objective. Data are expressed as the ratio between the number of TUNEL-positive nuclei and the total number of visual fields. Kidney sections from 17 to 20 mice per group were used for this analysis. The numbers of mice per group were as follows: *Nherf1* KO male vehicle, $n = 7$; WT male vehicle, $n = 10$; WT female vehicle, $n = 10$; *Nherf1* KO female vehicle, $n = 10$; WT male cisplatin, $n = 10$; WT female cisplatin, $n = 10$; *Nherf1* KO male cisplatin, $n = 10$; and *Nherf1* KO female cisplatin, $n = 10$.

Protein Determination and Immunoblots

Kidney cortex homogenates were made as previously described.[17] Protein concentration was measured by the bicinchoninic acid method (Sigma-Aldrich) using bovine serum albumin as a standard. Kidney homogenates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with 5% (w/v) dried milk in Tris-buffered saline with 0.5% Tween 20 (TTBS) for 30 minutes to decrease nonspecific antibody binding. Membranes were incubated overnight at 4°C with primary antibodies diluted (1:1000) in 5% milk. Membranes were washed with TTBS and incubated for 2 hours with horseradish peroxidase–conjugated secondary antibodies diluted in TTBS and 5% milk. Chemiluminescence (Thermo Fisher Scientific) was utilized to detect bands and visualization via GeneSys software version 4.03(b) with a Pixi imaging system (Syngene, Bangalore, India). Densitometric analysis was performed via ImageJ software version 1.53a (NIH, Bethesda, MD; *http://imagej.nih.gov.ij*). For densitometric analysis, specific protein expression was normalized to densitometry values for glyceraldehyde-3-phosphate dehydrogenase in each lane. The numbers of mice per group were as follows: WT male vehicle, $n = 4$; WT female vehicle, $n = 4$; *Nherf1* KO male vehicle, $n = 4$; *Nherf1* KO female vehicle, $n = 4$; WT male cisplatin, $n = 4$; WT female cisplatin, $n = 4$; *Nherf1* KO male cisplatin, $n = 4$; and *Nherf1* KO female cisplatin, $n = 4$.

Statistical Analysis

Data are expressed as means ± SEM. Three-way analysis of variance was used to compare the different treatment groups. Ordinal regression was performed for the semiquantitative scoring. All statistical analysis was conducted

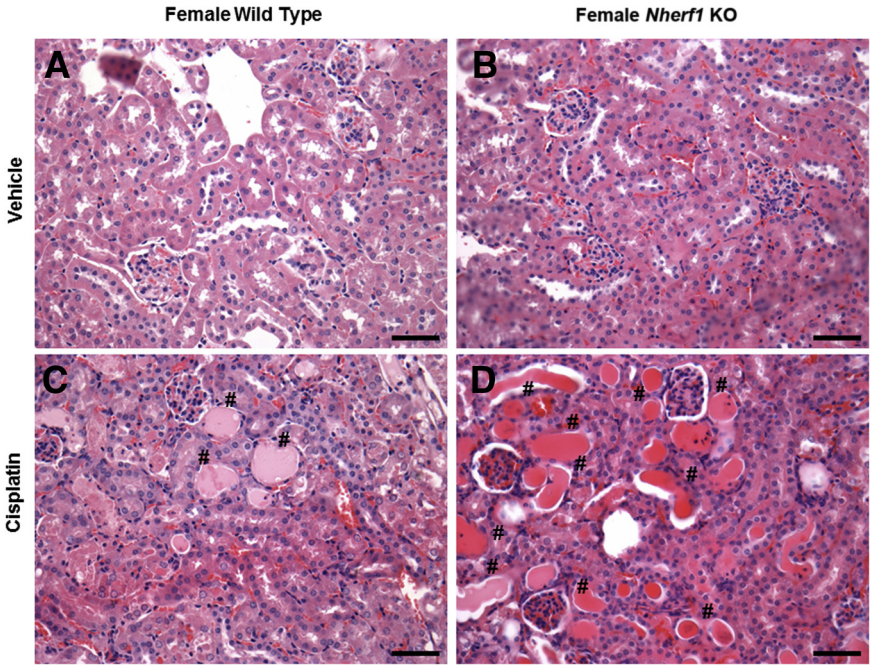

Figure 3 The histologic effect of cisplatin on hematoxylin and eosin (H&E) in female wild-type (WT) and *Nherf1* knockout (KO) mice. Representative photomicrographs of H&E staining of whole kidney sections embedded in paraffin and cut at 5 μm thick either transversely or longitudinally from cisplatin- and vehicle-treated WT and *Nherf1* KO female animals. **A**–**D:** A vehicle-treated female WT kidney (**A**), a vehicle-treated female *Nherf1* KO kidney (**B**), a cisplatin-treated female WT kidney (**C**), and a cisplatin-treated female *Nherf1* KO kidney (**D**). **Pound signs** indicate tubular casts. Scale bars: 50 μm. Original magnification, ×40.

with IBM SPSS Statistics version 24 (IBM, Armonk, NY). *P* values of <0.05 were considered statistically significant.

Results

Cisplatin treatment was associated with significantly increased BUN level and urine NGAL protein in *Nherf1* KO mice in comparison to cisplatin-treated WT.

To determine whether cisplatin treatment affected kidney function in *Nherf1* KO mice to a greater degree than in WT mice, male and female 2-month–old mice were treated with cisplatin to induce AKI and sacrificed after 72 hours. Blood samples were collected and plasma was separated for BUN measurement as described in *Materials and Methods*. There were no significant differences in baseline BUN between vehicle-treated WT and *Nherf1* KO mice, regardless of sex (Figure 1A). With cisplatin treatment, both WT mice (male, 93.0 ± 8.4 mg/dL; female, 101.9 ± 17.9 mg/dL) and *Nherf1* KO mice (male, 139 ± 11.4 mg/dL; female, 163.8 ± 33.4 mg/dL) had significant increases in BUN compared to vehicle-treated WT mice (male, 28.5 ± 3.2 mg/dL; female,

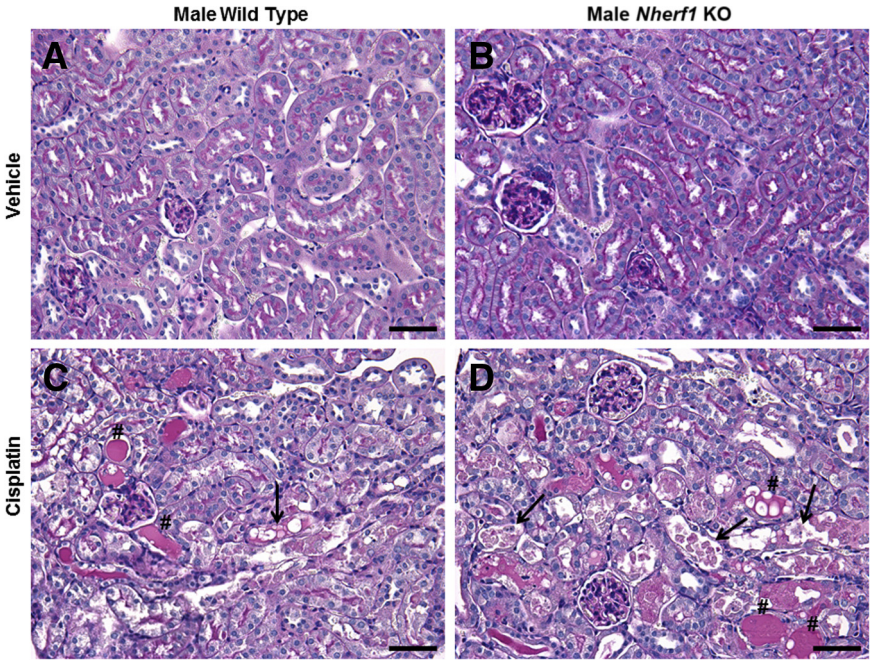

Figure 4 The histologic effect of cisplatin on periodic acid-Schiff (PAS) in male wild-type (WT) and *Nherf1* knockout (KO) mice. Representative photomicrographs of PAS staining of whole kidney sections embedded in paraffin and cut at 5 μm thick either transversely or longitudinally from cisplatin- and vehicle-treated WT and *Nherf1* KO male animals. A vehicle-treated male WT kidney (**A**), a vehicle-treated male *Nherf1* KO kidney (**B**), a cisplatin-treated male WT kidney (**C**), and a cisplatin-treated male *Nherf1* KO kidney (**D**). **Arrows** indicate acute tubular necrosis; **pound signs** indicate tubular casts. Scale bars: 50 μm. Original magnification, ×40.

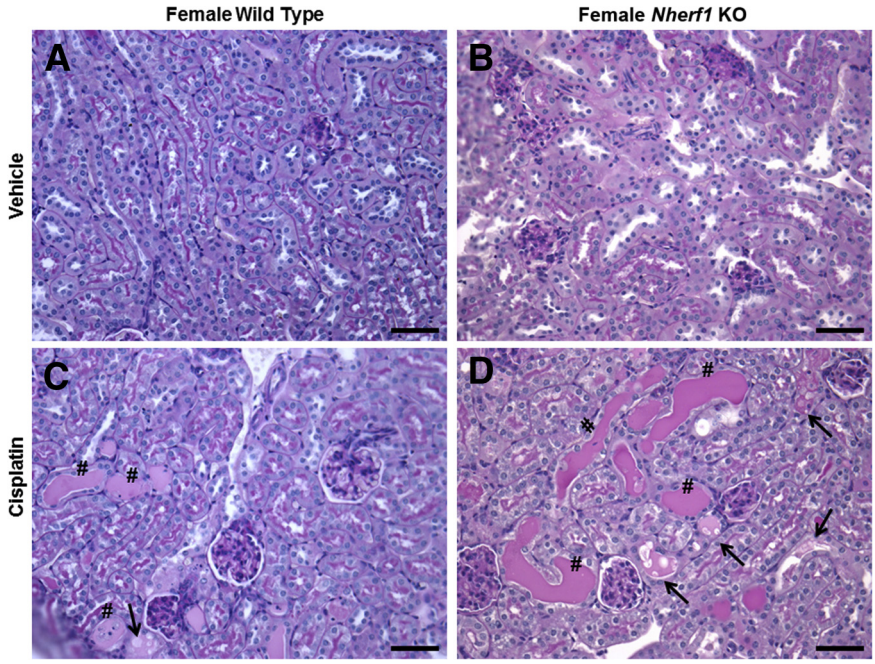

Figure 5 The histologic effect of cisplatin on periodic acid-Schiff (PAS) in female wild-type (WT) and *Nherf1* knockout (KO) mice. **A–D:** Representative photomicrographs of PAS staining of whole kidney sections embedded in paraffin and cut at 5 μm thick either transversely or longitudinally from cisplatin- and vehicle-treated WT and *Nherf1* KO female animals. A vehicle-treated female WT kidney (**A**), a vehicle-treated female *Nherf1* KO kidney (**B**), a cisplatin-treated female WT kidney (**C**), and a cisplatin-treated female *Nherf1* KO kidney (**D**). **Arrows** indicate acute tubular necrosis; **pound signs** indicate tubular casts. Scale bars: 50 μm. Original magnification, ×40.

25.2 ± 3.9 mg/dL) and vehicle-treated *Nherf1* KO mice (male, 31.5 ± 3.7 mg/dL; female, 38.0 ± 2.2 mg/dL) ($P < 0.001$) (Figure 1A). However, a statistically significant increase in BUN level was observed in cisplatin-treated *Nherf1* KO compared to cisplatin-treated WT mice ($P < 0.05$) (Figure 1A). No statistical differences between sexes of the mice or levels of BUN were found under baseline conditions or with cisplatin treatment (Figure 1A).

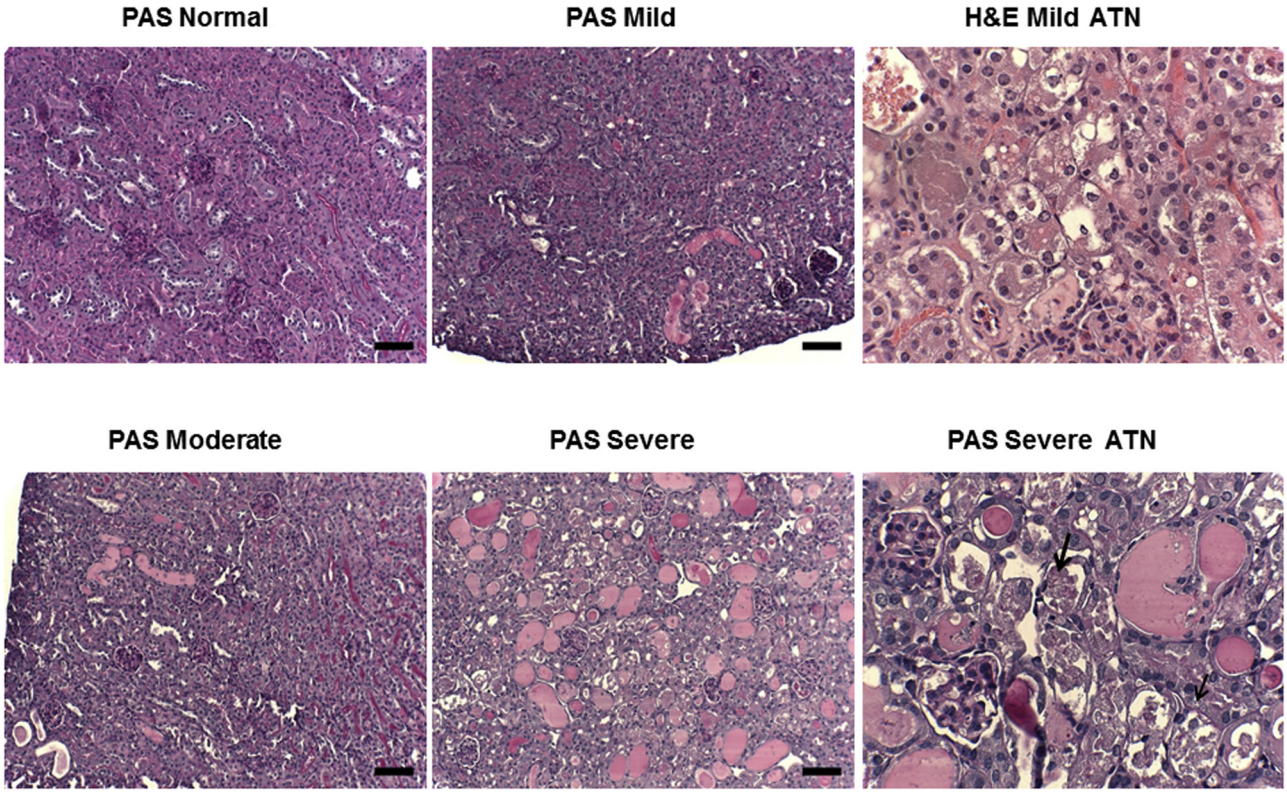

Figure 6 Examples of normal, mild, moderate, and severe injury for semiquantitative scoring. Representative hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining of whole kidney sections embedded in paraffin and cut at 5 μm thick either transversely or longitudinally from cisplatin- and vehicle-treated wild-type and *Nherf1* knockout male and female animals. Examples of normal, mild, moderate, severe injury, and acute tubular necrosis (ATN; **arrows**) scoring are shown. Scale bars: 50 μm. Original magnification: ×20 (**left** and **middle columns**); ×60 (**right column**)

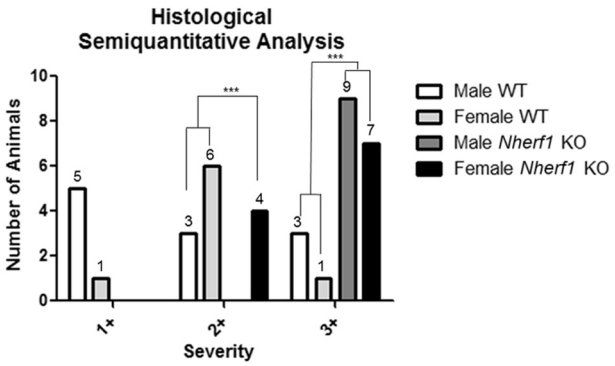

Figure 7 Histologic semiquantitative scoring of cisplatin-treated wild-type (WT) and *Nherf1* knockout (KO) mice. Ordinal regression analysis of the semiquantitative scoring. Data are expressed as the numbers of animals with 1+, 2+, and 3+ severity scores. $n = 8$ (WT female); $n = 9$ (*Nherf1* KO male); $n = 11$ (WT male and *Nherf1* KO female). ***$P < 0.001$.

Urine samples were collected and NGAL was measured as described in *Materials and Methods*. With cisplatin treatment, both WT mice (male, 3.4 ± 1.8 μg/mL; female, 2.5 ± 0.4 μg/mL) and *Nherf1* KO mice (male, 21.5 ± 13.9 μg/mL; female, 82.8 ± 33.1 μg/mL) had significant increases in NGAL protein compared to vehicle-treated WT mice (male, 0.03 ± 0.01 μg/mL; female, 0.02 ± 0.006 μg/mL) and vehicle-treated *Nherf1* KO mice (male, 0.01 ± 0.006 μg/dL; female, 0.02 ± 0.005 μg/mL) ($P < 0.01$) (Figure 1B). However, a statistically significant increase in NGAL protein level was observed in cisplatin-treated *Nherf1* KO compared to cisplatin-treated WT mice ($P < 0.05$) (Figure 1B). A significant decrease in NGAL protein was found in vehicle-treated *Nherf1* KO mice compared to vehicle-treated WT mice ($P < 0.05$) (Figure 1B). No statistical differences between sexes of the mice and NGAL protein were found under baseline conditions or with cisplatin treatment (Figure 1B).

Effects of Cisplatin Treatment on *Kim1* mRNA Expression in WT and *Nherf1* KO Mice

Kim1 mRNA expression was not significantly different between WT and *Nherf1* KO vehicle-treated mice (Figure 1C). However, *Kim1* mRNA expression was significantly increased in both cisplatin-treated WT mice (male, 2363.5 ± 1182.1-fold of control; female, 724.6 ± 386.3-fold of control) and *Nherf1* KO mice (male, 4514.2 ± 3157.4-fold of control; female, 1175.2 ± 295.7-fold of control) ($P < 0.05$) in comparison to vehicle-treated WT mice (male, 0.9 ± 0.4-fold of control; female, 0.5 ± 0.2-fold of control) and *Nherf1* KO mice (male, 3.3 ± 0.9-fold of control; female, 1.6 ± 0.7-fold of control) (Figure 1C). There was no significant difference in the kidney expression of *Kim1* mRNA with cisplatin treatment between WT and *Nherf1* KO mice; no statistical difference between sexes or *Kim1* mRNA expression was found.

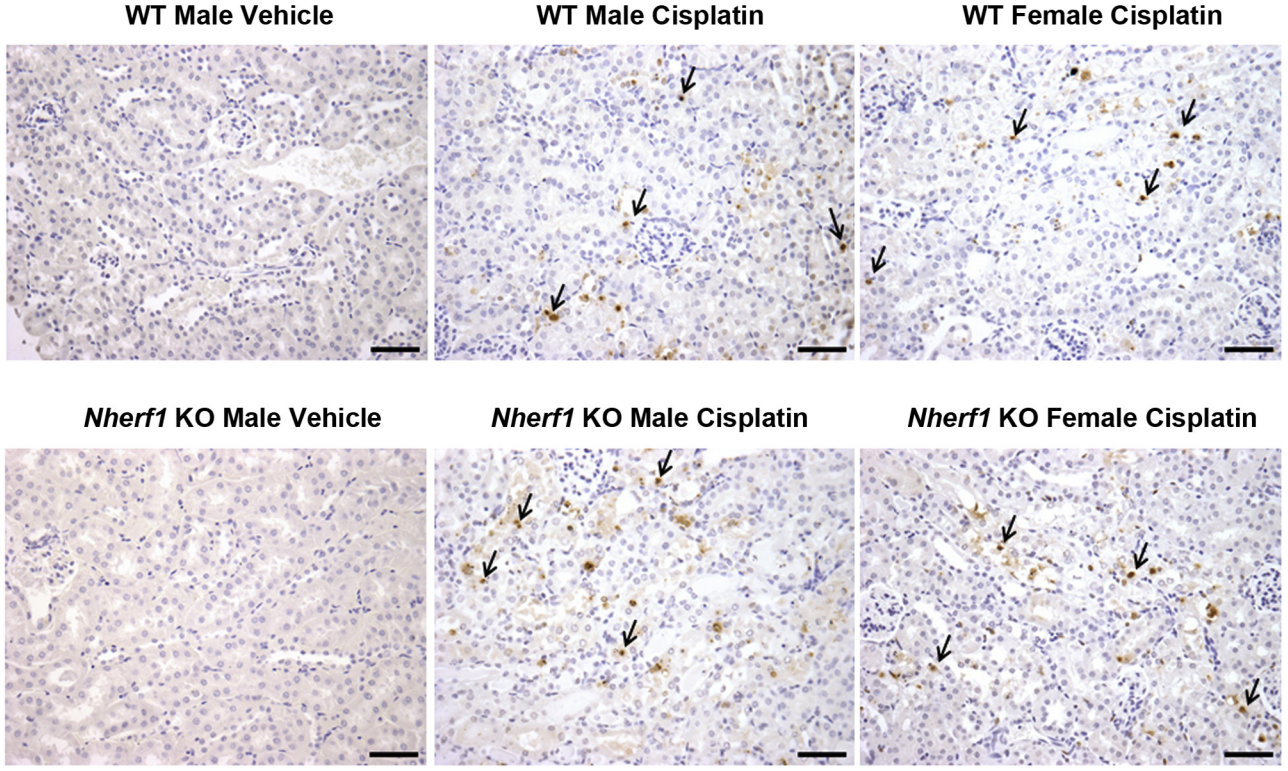

Figure 8 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay photomicrographs of vehicle- and cisplatin-treated wild-type (WT) and *Nherf1* (KO) mice. Representative photomicrographs of vehicle- and cisplatin-treated WT and *Nherf1* KO male and female mice and TUNEL-positive nuclei (**arrows**). TUNEL-positive nuclei were seen in the kidney of animals that received cisplatin. Scale bars = 50 μm.

Severity of Renal Injury in Cisplatin-Treated *Nherf1* KO Mice

H&E and PAS staining of the kidneys in cisplatin-treated *Nherf1* KO mice showed that the injury was predominantly cortical (Figures 2, 3, 4, and 5). H&E (Figures 2 and 3) and PAS (Figures 4 and 5) staining revealed that kidneys from vehicle-treated WT and *Nherf1* KO mice were histologically similar, with no structural differences (Figures 2, 3, 4, and 5). As has been observed previously in H&E and PAS staining,[20,21] cisplatin treatment in WT mouse kidneys resulted in visible histologic damage, primarily in the proximal tubules (Figures 2, 3, 4, and 5). This damage was manifested by severe cell damage, casts, atrophy, brush border membrane sloughing, and acute tubular necrosis. With cisplatin treatment, *Nherf1* KO mice had a visibly greater degree of histologic damage seen with both H&E (Figures 2 and 3) and PAS (Figures 4 and 5) staining.

To quantitate the differences in the degree of histologic damage between cisplatin-treated WT and *Nherf1* KO mice, kidney sections were sent to a pathologist (S.C.) for semi-quantitative scoring in a blinded study, as described in *Histology and Semiquantitative Scoring* (Figure 6). The histology of all vehicle-treated mice, regardless of genotype (WT or *Nherf1* KO), was assessed as normal (score, 0), without injury. The severities of injury in the cisplatin-treated WT kidneys were graded as mild (1+) in 6 mice, moderate (2+) in 9, and severe (3+) in 4 (Figure 7). In contrast, the injury in majority ($n = 16$) of kidneys from the cisplatin-treated *Nherf1* KO animals fell under the category of severe (3+), and none of the cisplatin-treated *Nherf1* KO kidneys were graded as having mild injury (Figure 7). The vehicle-treated kidneys in the WT ($n = 20$) and *Nherf1* ($n = 22$) groups are not shown in Figure 7. The mean injury scores were 1.89 in the WT group and 2.8 in the *Nherf1* KO group ($P < 0.001$) (Figure 7).

Degrees of Apoptosis in Cisplatin-Treated WT and *Nherf1* KO Mice

To compare the degree of apoptosis in WT versus *Nherf1* KO mouse kidneys, two different assays for apoptosis were performed, TUNEL and caspase 3 cleavage. Kidney sections were subjected to TUNEL analysis (Figures 8 and 9). TUNEL assay analysis showed significantly increased degrees of apoptosis (measured as the number of positive nuclei per number of visual fields) in both cisplatin-treated *Nherf1* KO mice (male, 12.9 ± 2.5; female, 11.3 ± 3.2) and WT mice (male, 10.6 ± 1.4; female, 8.3 ± 1.4) in comparison to vehicle-treated *Nherf1* KO mice (male, 0.2 ± 0.06; female, 0.2 ± 0.05) and WT mice (male, 0.1 ± 0.05; female, 0.03 ± 0.02) ($P < 0.001$) (Figure 9). There were no significant differences in the levels of apoptosis between the cisplatin-treated WT and *Nherf1* KO mice or between the vehicle-treated WT and *Nherf1* KO mice (Figure 9), despite a greater degree of tubular necrosis observed in the kidneys of the cisplatin-treated *Nherf1* KO mice (Figure 4).

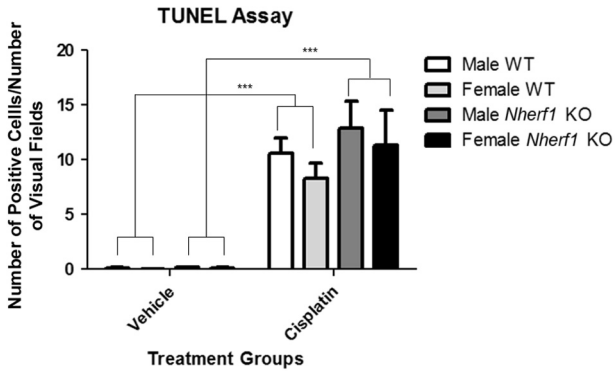

Figure 9 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay of vehicle- and cisplatin-treated wild-type (WT) and *Nherf1* knockout (KO) mice. Quantitation of TUNEL-positive nuclei was performed in a blinded fashion (A.B.-S.) and only positively stained nuclei exhibiting characteristics of apoptotic nuclei were included. Twenty to 30 randomly chosen visual fields were observed under a ×40 objective as described in the *Materials and Methods*. Data are expressed as mean ± SEM ratios between the number of TUNEL-positive nuclei and the total number of visual fields. $n = 7$ (*Nherf1* KO male vehicle); $n = 10$ (WT male vehicle, WT female vehicle, *Nherf1* KO female vehicle, WT male cisplatin, WT female cisplatin, *Nherf1* KO male cisplatin, and *Nherf1* KO female cisplatin). ***$P < 0.001$ (three-way analysis of variance).

To confirm the TUNEL assay findings, cleaved caspase 3, the activated terminal component of the caspase cascade, was analyzed via Western blot analysis. A significant increase in cleaved caspase 3 expression was demonstrated in cisplatin-treated WT and *Nherf1* KO mouse kidneys compared to their respective vehicle-treated controls ($P < 0.01$) (Figure 10), but the difference in cleaved caspase 3 expression between cisplatin-treated WT and *Nherf1* KO mouse kidneys was not significant. Interestingly, a significant sex difference was documented, with an increase in cleaved caspase 3 expression in female vehicle-treated compared to male vehicle-treated mouse WT and KO kidneys ($P < 0.05$) (Figure 10).

Discussion

The goal of this study was to determine whether NHERF1 loss results in increased susceptibility to cisplatin-induced AKI. This is the first study to investigate the role of NHERF1 in cisplatin-induced AKI, and the first to show that *Nherf1* KO mice have increased susceptibility to cisplatin-induced AKI.

The baseline BUN of vehicle-treated *Nherf1* KO mice was comparable to that in vehicle-treated WT mice, indicating that renal function is similar under baseline conditions. *Nherf1* KO mice exhibit significant differences in the expression of brush border membrane proteins of the small intestine,[22] suggesting that *Nherf1* KO mice might experience chronic volume depletion due to poor gastrointestinal solute absorption, which could be a mechanism for increased susceptibility to toxin-induced AKI. However, the similar baseline BUN levels

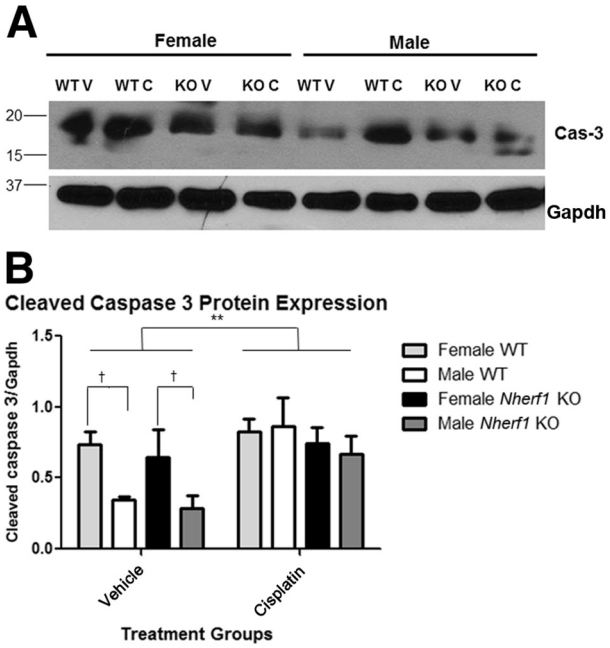

Figure 10 Effect of cisplatin on cleavage of caspase 3 in wild-type (WT) and *Nherf1* knockout (KO) mice. **A:** Representative blots of cleaved caspase (Cas)-3 and glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Kidney homogenates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Quantitation was performed as described in *Materials and Methods* of cleaved caspase 3 (both 17 and 19 kDa bands). **B:** A representative blot from four independent experiments. Data are expressed as means ± SEM (**B**). $n = 4$ mice per treatment group. $^{**}P < 0.01$ cisplatin treatment compared to vehicle alone; $^{\dagger}P < 0.05$ females compared to males alone (three-way analysis of variance). c., cleaved; KOC, knockout, cisplatin; KOV, knockout, vehicle; WTC, wild-type, cisplatin; WTV, wild-type, vehicle.

coupled with those from prior studies showing similar blood pressure and urine sodium excretion[19] do not suggest chronic volume depletion in the *Nherf1* KO animals, thus decreasing the likelihood of this mechanism in the increased susceptibility of *Nherf1* KO mice to cisplatin-induced AKI. Cisplatin increased BUN and NGAL in both WT and *Nherf1* KO mice, with the cisplatin-treated *Nherf1* KO mice having significantly higher BUN and NGAL than cisplatin-treated WT mice. These results are consistent with a significantly greater decrease in renal function in *Nherf1* KO animals when challenged with cisplatin.

The findings on histologic comparison of the kidneys of WT and *Nherf1* KO animals support the biochemical data. The kidneys of vehicle-treated WT and *Nherf1* KO mice were morphologically and structurally similar. Cisplatin treatment in *Nherf1* KO mice was associated with a greater severity of injury, and an overall severity score that was significantly higher than the score in WT mice. Furthermore, a significantly higher number of cisplatin-treated *Nherf1* KO mice were found to have the most severe injury (3+) compared to cisplatin-treated WT mice.

Kim-1, a sensitive marker of kidney tubular injury, was significantly increased with cisplatin treatment in WT and *Nherf1* KO mice. Kim-1 was not increased in baseline measurements in vehicle-treated WT and *Nherf1* KO mice, indicating that under baseline conditions, *Nherf1* KO mice do not have detectable levels of underlying injury. No significant difference in Kim-1 expression levels was found in cisplatin-treated WT and *Nherf1* KO mouse kidneys. This finding is ostensibly inconsistent with significant differences in histologic damage and BUN levels between WT and *Nherf1* KO mice treated with cisplatin. However, the histologic kidney sections of cisplatin-treated *Nherf1* KO mice showed a far greater degree of brush border membrane sloughing and tubular necrosis, which would decrease *Kim1* mRNA and protein expression.[23] A previous study evaluating the effect of necrosis on the expression of Kim-1 in the setting of proximal tubule injury in rats demonstrated that Kim-1 expression was present at higher levels in tubules that showed modest levels of necrosis, whereas Kim-1 expression was present at lower levels in tubules with severe levels of necrosis.[24] The investigators hypothesized that KIM-1 expression may be affected by phagocytic and repair mechanisms in response to injury and therefore is mainly found in nonlethally injured cells.[24]

Cisplatin induces renal proximal tubule cell death through both necrosis and apoptosis.[20,25,26] TUNEL analysis and cleavage of caspase 3 demonstrated that at 72 hours after cisplatin treatment, apoptosis was increased similarly in both WT and *Nherf1* KO mouse kidneys. Under baseline vehicle-treatment conditions, there was no detectable difference in apoptosis between WT and *Nherf1* KO mouse kidneys as measured by TUNEL assay. Overall, these data suggest that the greater degree of injury seen with cisplatin treatment in the *Nherf1* KO mice was a result of increased cell death via tubular necrosis when compared with the WT animals. Interestingly, there was a significant difference in baseline caspase 3 cleavage between male and female mice, with the female mice showing significantly higher levels of caspase 3 cleavage product, independent of genotype. The significance of this finding is unknown at this time, as no other sex differences related to kidney injury were found in this study.

This study demonstrates a novel role for NHERF1 in protection against cisplatin-induced AKI. NHERF1 has been identified as a trafficking protein and a scaffolding protein that is involved in the formation of multiprotein complexes. Additionally, NHERF1 has importance in metabolism.[27] Therefore, several potential mechanisms may explain the susceptibility to cisplatin, including mitochondrial dysfunction, changes in metabolic pathways, alterations in intracellular signaling,[28–30] decreased ATP production due to hypophosphatemia,[31] changes in renal handling of cisplatin,[32–35] and/or reduction in regeneration capabilities of proximal tubules. These findings have potential clinical significance. Aging rats (22 months) show a decrease in Nherf1 expression.[17] Clinically, the elderly population experience greater cancer prevalence and are more likely to develop AKI in response to insults such as that associated with cisplatin use.[36,37] Thus, in the elderly population, NHERF1 loss may occur and contribute to the risk for AKI. Potentially,

NHERF1 expression could be used as a biomarker for susceptibility to AKI or as a target for innovative therapies to protect against cisplatin-induced AKI.

Acknowledgments

We thank Susan Isaacs for expert technical support and the Division of Nephrology for financial support.

A.B-S. was the primary investigator on this project and performed all experiments and was the primary author of the manuscript; M.B. assisted technically with staining and imaging and edited the manuscript; C.C. assisted technically with experiments and edited the manuscript; T.D. assisted with animal treatments; K.B.G. assisted with tissue collection; S.J.K provided editing assistance with the manuscript; B.C. provided technical and intellectual assistance with experiments; L.S. provided technical and intellectual assistance with KIM-1 experiments; M.A.D. performed ELISA assay for NGAL; M.R. performed experiments for cleavage of caspase 3; M.B. provided expert statistical analysis for all experiments; S.C. provided all semiquantitative scoring of kidney sections from the experiments; E.D.L. is the mentor of the primary investigator, provided intellectual/conceptual input into experimental design and results analysis, and edited the manuscript; E.D.L. is the guarantor of this work and had full access to all of the data in this study and takes full responsibility for the integrity of the data and the accuracy of the data analysis.

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