Essential Role of X-Box Binding Protein-1 during Endoplasmic Reticulum Stress in Podocytes

Hossam Hassan,* Xuefei Tian,† Kazunori Inoue,† Nathan Chai,† Chang Liu,† Keita Soda,† Gilbert Moeckel,‡ Alda Tufro,* Ann-Hwee Lee,§ Stefan Somlo,† Sorin Fedeles,† and Shuta Ishibe†

*Department of Pediatrics, †Internal Medicine, and ‡Pathology, Yale University School of Medicine, New Haven, Connecticut, and §Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, New York

ABSTRACT

Podocytes are terminally differentiated epithelial cells that reside along the glomerular filtration barrier. Evidence suggests that after podocyte injury, endoplasmic reticulum stress response is activated, but the molecular mechanisms involved are incompletely defined. In a mouse model, we confirmed that podocyte injury induces endoplasmic reticulum stress response and upregulated unfolded protein response pathways, which have been shown to mitigate damage by preventing the accumulation of misfolded proteins in the endoplasmic reticulum. Furthermore, simultaneous podocyte-specific genetic inactivation of X-box binding protein-1 (Xbp1), a transcription factor activated during endoplasmic reticulum stress and critically involved in the untranslated protein response, and Sec63, a heat shock protein-40 chaperone required for protein folding in the endoplasmic reticulum, resulted in progressive albuminuria, foot process effacement, and histology consistent with ESRD. Finally, loss of both Sec63 and Xbp1 induced apoptosis in podocytes, which associated with activation of the JNK pathway. Collectively, our results indicate that an intact Xbp1 pathway operating to mitigate stress in the endoplasmic reticulum is essential for the maintenance of a normal glomerular filtration barrier.


Nephrotic syndrome is characterized by massive protein loss in the urine due to disruption of the glomerular filtration barrier. Podocytes are terminally differentiated glomerular epithelial cells, with interdigitated foot processes required to maintain the integrity of this barrier. Podocytes can respond to injury and various insults by invoking an adaptive mechanism recognized as the unfolded protein response (UPR), which attempts to mitigate damage by governing the accumulation of misfolded proteins in the endoplasmic reticulum (ER).1–3 Nevertheless, severe or protracted periods of ER stress can result in the activation of pro-apoptotic pathways. Three major “sensor” genes in the mammalian UPR pathway that are activated during ER stress include activating transcription factor-6 (ATF6), inositol-requiring enzyme-1 (IRE1), and protein kinase RNA-like ER kinase (PERK). Association with GRP78/binding immunoglobulin protein (BiP), a key ER regulatory protein usually keeps these genes inactive in quiescent cells.4 Recent evidence has suggested a robust increase in podocyte BiP due to ER stress caused by Pierson syndrome (laminin mutations), Alport syndrome, thin membrane basement disease (collagen IV mutations), Heymann nephritis, puromycin-induced nephrosis, and mesangioproliferative GN.5–8

Therefore, in the current study, we first verified that treatment of cultured podocytes with
tunicamycin, an agent that inhibits N-glycosylation of proteins in the ER, results in an increase in ER stress and activation of the UPR. This effect was further validated in vivo by glomeruli isolated from mice injected with rabbit-anti-mouse glomerular basement membrane nephrotoxic sera (NTS). To further address the role of ER stress and the UPR, we used a conditional gene knockout (KO) strategy by inactivating Sec63,8–11 a protein essential for folding, and X box-binding protein-1 (XBP1), a downstream transcription factor activated by ER stress due to splicing by activated IRE1α.12,13 Analysis of the KO mice demonstrated robust progressive proteinuria associated with severe foot process effacement. Furthermore, we showed that the podocyte-specific loss of Sec63 and Xbp1 (Pod-Sec63/Xbp1 DKO) results in podocyte depletion, which may be due to the increased activation of the c-Jun N-terminal kinase (JNK) pathway. Collectively, our results indicate that glomerular injury induces ER stress and the UPR, whose intact functions are required to maintain a normally functioning glomerulus.

RESULTS

ER Stress and UPR Are Elicited in Podocytes Following Tunicamycin Treatment

We initially revisited the role of ER stress in enriched primary podocytes through treatment with tunicamycin, an agent that inhibits N-glycosylation of proteins in the ER and provokes ER stress.8 Podocytes treated with tunicamycin displayed a noticeable increase in the expression of ER stress markers K-lysine, D-Aspartic Acid, E-Glutamic Acid, L-Leucine (KDEL) and BiP (GRP78) as demonstrated by western blotting (Figure 1A, quantified in Figure 1B) and immunofluorescence (Figure 1C). Because ER stress can induce the UPR response, we next examined the major arms of UPR pathways. In primary podocytes treated with tunicamycin, we observed an increase in the

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**Figure 1.** ER stress and activation of UPR response arms are observed in enriched primary podocytes treated with tunicamycin. (A) Enriched podocytes treated and not treated with tunicamycin (2 mg/ml) for 24 hours demonstrate increased expression of ER stress proteins KDEL and GRP78. (B) Quantification of immunoblots in part A. The expression levels of GRP78 and KDEL were normalized to GAPDH levels. *P<0.001; n=3 independent experiments. (C) Enriched primary podocytes treated and not treated with tunicamycin (2 mg/ml) for 8 and 24 hours demonstrate increased immunoreactivity to KDEL and GRP78. Scale bar=2 μm. (D) Enriched primary podocytes treated and not treated with tunicamycin (2 mg/ml) for 24 hours immunoblotted for ATF6α (N), p-elF2α, elF2α, p-PERK, PERK, XBP1s, and GAPDH. (E) Quantification of immunoblots in part D. The expression levels of ATF6α (N), p-elF2α, p-PERK, and XBP1s were normalized to GAPDH levels. *P<0.001; n=3 independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
activation of ATF6α, PERK, and IRE1 cleavage product XBP1 (Figure 1D and quantified in Figure 1E).

**ER Stress and the UPR Are Elicited in Podocytes following Nephrotoxic Serum Administration**

To validate our *in vitro* findings we injected mice with rabbit–anti-mouse glomerular basement membrane (NTS) to determine whether ER stress occurred *in vivo*. Following NTS-induced injury, Western blot of enriched glomerular lysates isolated by Percoll gradient revealed robust increases in ER stress proteins KDEL and BiP (GRP78) (Figure 2A; quantified in Figure 2B). Because GRP78 is a major ER chaperone cooperatively regulated by all three UPR branches,14 we hypothesize that the glomerular injury triggers UPR activation to

**Figure 2.** ER stress and activation of UPR response arms are observed in glomeruli isolated from NTS-treated mice. (A) Mice glomerular extracts isolated at 7 days after treatment or no treatment with NTS demonstrate increased expression of ER stress proteins KDEL and GRP78. (B) Quantification of representative blots in part A. The expression levels of KDEL and GRP78 were normalized to β-actin levels *P*<0.001; *n*=3 independent experiments. (C) Glomerular cross-sections from mice treated or not treated with NTS at 2 and 7 days after administration demonstrate increased immunoreactivity to KDEL and GRP78 (green) that partially colocalizes with nephrin. Scale bar=10 μm. (D) Isolated glomerular extracts from mice treated or not treated with NTS at 7 days after administration immunoblotted for ATF6α (N), p-eIF2α, eIF2α, p-PERK, PERK, XBP1s, and β-actin. (E) Quantification of immunoblots in part D. The expression levels of ATF6α (N), p-eIF2α, p-PERK, and XBP1s were normalized to β-actin levels. *P*<0.001; *n*=3 independent experiments. (F) Dilated endoplasmic reticulum (arrowheads) is observed in podocyte cross-sections visualized by electron microscopy. Scale bar=200 nm.
counteract ER stress. The validity of our model was further emphasized by immunofluorescence of kidney sections (Figure 2C). Glomeruli isolated from NTS-treated mice also demonstrated a striking increase in the activation of the three arms, which sense ER stress (Figure 2D; quantified in Figure 2E). Because immunoreactivity increased for the ER stress proteins (Figure 2C), we next measured ultrastructural changes in the ER using transmission electron microscopy (TEM). Morphologic analysis revealed ER distention and dilatation in podocytes from mice that were treated with NTS (Figure 2F).

**Podocyte Specific Ablation of Sec63 Induces Xbp1 Splicing**

Because the UPR response sensors were highly activated following glomerular injury, we next turned our attention toward ablating one of the UPR response arms using a conditional KO strategy. We generated a podocyte-specific KO of Xbp1 by crossing a previously described Xbp1fl/fl mouse with a mouse expressing Cre-recombinase under the podocyte promoter, podocin. Podocyte-specific Xbp1 KO mice were born in the normal Mendelian frequency and displayed no histologic evidence of glomerular injury up to 1 year of age (Supplemental Figure 1A). To induce ER stress genetically, we ablated Sec63, an ER chaperone protein associated with the Sec61 translocon complex, in a podocyte-specific manner. These mice also developed normally without evidence of proteinuria or abnormalities in kidney histologic features (Supplemental Figure 1, A and B). However, loss of Sec63 in podocytes activated ER molecular chaperone proteins, KDEL and GRP78 (Supplemental Figure 2, A and B), as well as induced Xbp1 splicing (Figure 3A) and nuclear staining (Figure 3D, middle panel), suggesting activation of IRE1α, a critical regulator of the UPR arm. Furthermore, the podocytes isolated from the Sec63 KO mouse revealed the activation of XBP1 target genes (Supplemental Figure 2C). Next, we examined the effect of loss of Sec63 and XBP1 simultaneously in podocytes (Figure 3B). Tail genotyping by PCR verified the presence of both Sec63 and Xbp1 alleles (Figure 3C), and loss of Sec63 and XBP1 expression was confirmed by Western blot on purified podocytes harvested from P21 mice (Figure 3F). Loss of podocyte XBP1 and Sec63 immunoreactivity was also observed (Figure 3, D and E) in the mutant mice.

**DISCUSSION**

During cell injury or stress, the ER tries unceasingly to assemble properly folded proteins to maintain homeostasis. Yet when the ER is overwhelmed, creating a disparity in the ratio of misfolded to folded proteins, a mechanism known as the
UPR intercedes to reduce protein translation and induces molecular chaperones responsible for protein folding. This fundamental machinery is also elicited in podocytes; previous reports have revealed increased expression of molecular chaperones and the UPR in vitro after albumin administration and in vivo after experimental glomerular injury in rodents. Furthermore, upregulation of ER stress proteins in human kidney biopsy specimens taken from patients harboring laminin and collagen IV mutations, patients with underlying diabetic nephropathy, and patients with
FSGS\textsuperscript{28,29} suggests the fundamental role of the UPR in podocyte biology. The GRP78 activation observed in a variety of glomerular diseases is likely an adaptive cellular process, which meets the demands of a pathologic condition and may arise irrespective of disease progression.

In this study, we support our hypothesis by detecting enhanced ER stress chaperones GRP 78 and KDEL, and increased activation of the UPR stress response arms (ATF6, Xbp1 splicing through IRE1\textsubscript{a}, and eIF2\textsubscript{a} phosphorylation by PERK) following both tunicamycin administration in podocytes and NTS injection in mice. To further address the importance of the UPR pathway, we demonstrate how genetic ablation of Sec63 and Xbp1 results in the loss of integrity of the kidney permeability barrier, as evidenced by severe progressive proteinuria and foot process effacement. To our knowledge, this is the first report providing genetic evidence in podocytes that loss of an intact UPR response during ER stress accounts for the striking phenotypes such as those observed in

**Figure 4.** Pod-Sec63/Xbp1 DKO mice demonstrate albuminuria, glomerulosclerosis, and kidney failure. (A) SDS-PAGE (Coomassie blue staining) of standard BSA (numbers indicate µg) and of urine from Pod-Sec63/Xbp1 DKO mice demonstrates albuminuria at 2, 6, and 12 months of age compared with littermate controls. Equal volumes of standard BSA and urine (2 µl) were loaded in each lane. (B) Quantification of urinary albumin normalized to creatinine at 2 weeks, 2 months, 6 months, and 12 months of age. *P<0.001; n=5 mice. (C) Pod-Sec63/Xbp1 DKO mice demonstrate progressive weight loss starting after 6 months, relative to littermate controls. *P<0.001; n=5 mice. (D) Pod-Sec63/Xbp1 DKO mice demonstrate elevated plasma creatinine at 6 months of age compared with littermate controls. *P<0.001; n=4 mice. (E) Representative light microscopy images (hematoxylin-eosin [H&E], periodic acid-Schiff [PAS], and trichrome) of glomeruli from Pod-Sec63/Xbp1 DKO mice at indicated time points revealing histologic evidence of diffuse glomerulosclerosis by 12 months of age. Scale bar=25 µm. (F) Proteinaceous casts (arrowhead), dilated tubules, and interstitial fibrosis are observed in Pod-Sec63/Xbp1 DKO mice at 6 and 12 months of age. Scale bar=50 µm. (G) Quantification of glomerulosclerosis at 2, 6, and 12 months of age. *P<0.001 (6 months); *P<0.001 (12 months); n=3 different mice were used in independent experiments. (H) Quantification of interstitial fibrosis at 2, 6, and 12 months of age. *P<0.001 (6 months); *P<0.001 (12 months); n=3 different mice were used in independent experiments. (Black columns represent DKO, red columns represent control.)
the Pod-Sec63/Xbp1 DKO mice. Supporting these findings, deletion of Sec63 alone in podocytes activated ER stress chaperone proteins, but did not bring forth an overt phenotype. This may be accounted for by an intact UPR response. Examining the other UPR arms, PERK, and ATF6 by podocyte-specific genetic mice models of disease will further address its importance in podocyte biology. Although an intact UPR following ER stress appears critical, this does not eliminate contributions of other quality control measures governed by ER-associated degradation and autophagy. Autophagy, which also removes misfolded proteins, is important for podocyte health, and the role of ER-stress induced autophagy appears to be cell specific. Both the prevention and induction of cell death has been reported. However, our Pod-Sec63/Xbp1 DKO mice did not reveal a significant increase in autophagosomes (data not shown) compared with littermate controls by ultrastructural examination. Further studies evaluating the role of ERAD proteins and autophagy following ER stress in podocyte biology would be of great interest.

Prolonged ER stress resulting in the inability of the UPR to attenuate misfolded protein synthesis has been demonstrated to induce apoptosis. Staining with TUNEL revealed an increase in apoptosis, which may be due to the induction of the JNK-caspase pathway. Further studies with podocyte-specific ablation of JNK and/or treatment with JNK inhibitor is needed to elucidate the contribution of this pathway in podocyte apoptosis following UPR activation because inhibition of JNK also worsens albuminuria in diabetic models of nephropathy. This may also account for the reduction in podocyte number in the Pod-Sec63/Xbp1 DKO mice. Because WT1 regulates the expression of the slit-diaphragm protein nephrin, the modest reduction in nephrin expression in the Pod-Sec63/Xbp1 DKO glomeruli may further account for our observed phenotype. Interestingly, by 6 months of age, the Pod-Sec63/Xbp1 DKO mice had a >20% reduction in podocyte number calculated by WT1 staining, and by 1 year of age there was a 56% reduction in histologic findings of progression from focal sclerosis to global sclerosis. Our findings support studies demonstrating that the extent of podocyte loss correlates with the worsened pathologic findings observed.

In conclusion, our findings support the important role of ER stress following podocyte injury and further improves our understanding of how the podocyte may defend itself during stress through the activation of the UPR, which, when compromised, can result in a defective glomerular filtration barrier.

**CONCISE METHODS**

**Antibodies**

Antibodies used in this study were as follows: rabbit anti-eIF2α antibody, rabbit anti-phospho eIF2α, rabbit anti-PERK, rabbit anti-phospho-PERK, mouse anti-phosphoJNK antibody, rabbit anti-PARP antibody, rabbit anti-cleaved caspase-3 antibody, and rabbit anti-histone 3 antibody, all purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti–XBP-1s antibody (Biolegend, San Diego, CA).
CA), mouse anti-GRP78 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-KDEL antibody (Enzo Life Sciences), rabbit anti-ATF6α (gift from Ann-Hwee Lee, Cornell University), mouse anti-β-actin, mouse anti-glyceraldehyde 3-phosphate dehydrogenase (Sangene Biotech), rabbit anti-Sec63 (Proteintech), Alexa Fluor 488 goat anti-rabbit IgG antibody, Alexa Fluor 488 goat anti-mouse IgG antibody, Alexa Fluor 594 goat anti-rabbit IgG antibody, Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 594 goat anti-guinea pig IgG antibody were purchased from Invitrogen. Guinea pig anti-nephrin antibody (Progen Biotechnik, Heidelberg, Germany) and rabbit anti-WT1 polyclonal antibody were purchased from Santa Cruz Biotechnology, and rabbit anti-nephrin antibody used for Western blotting was a gift from Dr. Yutaka Harita (University of Tokyo, Japan).

**Creation and Genotyping of Podocyte-Specific Sec63/Xbp1 KO Mice**

Xbp1 fl/fl mice were obtained from Dr. Ann Hwee Lee and Sec63 fl/fl mice were obtained from Dr. Stefan Somlo. These were mated with Podocin-Cre mice to generate a podocyte-specific DKO of Sec63 and Xbp1 (Pod-Sec63/Xbp1 DKO). Tail genotyping was performed by PCR using previously described protocols for Xbp112,13 and Sec63,9,11 Pod- Sec63/Xbp1 DKO mice (homozygous for the floxed Sec63 and Xbp1 alleles) were generated and analyzed.

**Figure 6.** Pod-Sec63/Xbp1 DKO mice glomeruli have increased apoptosis. (A) Representative image of glomerulus from control and Pod-Sec63/Xbp1 DKO mice kidneys stained with WT1, at 6 months of age. Scale bar=10 μm. (B) Representative images of glomerulus from control and Pod-Sec63/Xbp1 DKO mice kidneys stained with TUNEL at 6 month of age. Scale bar=10 μm. (C) Quantification of WT1-positive glomeruli at 2, 6, and 12 months of age from control and Pod-Sec63/Xbp1 DKO mice (black columns represent DKO, red columns represent control). *P<0.001; n=3. (D) Quantification of the percentage TUNEL-positive glomeruli at 2, 6, and 12 months of age from control and Pod-Sec63/Xbp1 DKO mice (black columns represent DKO, red columns represent control). *P<0.001; **P<0.05; n=3. (E) Glomerular lysates from control and Pod-Sec63/Xbp1 DKO mice immunoblotted with p-JNK and cleaved caspase 3. β-Actin is used as a loading control. (F) Glomerular lysates from control and Pod-Sec63/Xbp1 DKO mice immunoblotted with PARP1 reveals 89 kDa c-terminal cleavage product. Histone 3 and GAPDH are used as loading controls. (G) Quantification of the immunoblots in parts E and F. *P<0.001; n=3 independent experiments (black columns represent DKO, red columns represent control). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
floxed Xbp1 alleles with Podacin-Cre) and littermate controls (homozygous for Sec63 and Xbp1 alleles but lacking Podacin-Cre) were used in these experiments. All animal experiments were approved by the University Committee on the Use and Care of Animals Institutional Review Board at Yale University. All work was conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) guidelines for the care and use of experimental animals.

**Cell Culture**

Isolation of podocytes from day 1–3 Pod-Sec63/Xbp1 DKO and littermate control pups was performed as described previously in our laboratory. Glomeruli were harvested and enriched from mice kidney cortical tissue with 45% Percoll solution (GE Healthcare Bio-Sciences, Upplands, Sweden) as previously described. The kidneys were harvested immediately and washed in ice-cold Krebs-Henseleit saline solution (119 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 7 H2O, and 25 mM NaHCO3, pH 7.4). The renal cortex segments were dissected away and analyzed as specified in each experiment (8–24 hours).

**Western Blot**

Freshly isolated glomeruli and cultured primary podocytes were lysed into lysis buffer containing 50 mM Tris-HCl (pH 7.6), 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM MgCl2, and protease inhibitor cocktail (Roche Diagnostics). Protein concentrations were measured using Bio-Rad protein assay. Total protein samples were separated using 20% gradient SDS-PAGE gels, and transferred to the Immobilon poly-vinylidene fluoride membranes (EMD Millipore). The membrane was blocked with 3% nonfat milk or 3% BSA in 1× Tris-buffered saline and Tween 20 and incubated with the appropriate primary antibody at 4°C overnight. Following three washes with 1× Tris-buffered saline and Tween 20, the appropriate peroxidase-labeled anti-IgG secondary antibody (Bio-Rad) was added and signals were detected using enhanced chemiluminescence reagents (PerkinElmer). For quantification, densitometry was performed with ImageJ software (NIH).

**Kidney Histology, Immunofluorescence Staining of Kidney Tissues, and Quantification**

Mice were anesthetized by intraperitoneal injection of ketamine followed by perfusion fixation with 40 ml of 4% paraformaldehyde with or without 2% glutaraldehyde for immunofluorescence or TEM experiments, respectively.

For histology, kidney sections were sent to Yale Pathology Core Tissue Services for hematoxylin and eosin, periodic acid-Schiff, or Masson trichrome staining. For TEM, kidneys were postfixed with Palade osmium (4% OSO4 in double distilled water; pH 7.6). TEM was performed by the Cellular and Molecular Physiology Core Services at Yale.

For immunofluorescence, kidney cryosections were subjected to antigen retrieval at 95°C for 10 minutes in retrievagen A solution (pH 6.0) (BD Biosciences), followed by blocking with 3% BSA in 1× PBS for 1 hour at room temperature. Immunostaining was performed with the appropriate primary antibodies overnight at 4°C and Alexa Fluor 488 and/or 594 conjugated secondary antibodies at room temperature for 1 hour. Slides were mounted with Slowfade, containing 4,6-diamidino-2-phenylindole (Invitrogen). Images were acquired with the Carl Zeiss LSM 710 laser scanning confocal microscope using a 63× Plan Apo (numeric aperture=1.4) oil immersion objective for immunofluorescence analysis, and images were processed using ImageJ software (version 1.47H) or Adobe Photoshop CS4.

For quantitative analysis of kidney histologic findings, 50 full-sized glomeruli from each specimen were assessed on periodic acid-Schiff-stained sections, and the level of glomerulosclerosis in each glomerulus was semi-quantitatively scored as follows: 0=no sclerosis; 1=sclerosis of <10% of the of glomeruli; 2=sclerosis of 10%–25% of the glomeruli; 3=sclerosis of 25%–50% of the glomeruli; 4=sclerosis of >50% of glomeruli. To evaluate interstitial fibrosis, 20 fields for each section were assessed on Masson trichrome–stained sections. Semi-quantitative analysis in each field was assessed as follows: 0=no fibrosis; 1=fibrosis of <10% of areas; 2=fibrosis of 10%–25% of areas; 3=fibrosis of 25%–50% of areas; 4=fibrosis of >50% of areas. The averages of the glomerulosclerosis and interstitial fibrosis scores were calculated from the total evaluated glomeruli or interstitial lesions in each section. These microscopic evaluations were performed by Dr. Xuefei Tian without prior prejudicial information.

**Apoptosis Assay**

Apoptosis was detected in kidney cryosections from control and Pod Sec63/Xbp1 DKO mice by TUNEL assay using the In situ Cell Death Detection kit (Roche) as described by the manufacturer’s protocol. Glomerular apoptosis was identified as the number of glomeruli with at least one TUNEL-positive nuclei over the total number of glomeruli. All glomeruli from each kidney were accounted for, and three independent experiments were performed and quantified.

**Immunofluorescence Staining of Primary Podocytes**

Cultured isolated primary podocytes were fixed in 4% paraformaldehyde in 1× PBS, permeabilized with 0.1% Triton X-100 in 1× PBS for 10 minutes, blocked with 3% BSA, then incubated with the appropriate primary and secondary antibodies as described earlier.
Quantitative PCR Analysis

After kidney extraction and isolation of glomeruli, total RNA was extracted using an RNA extraction kit (Macherey-Nagel, Düren, Germany). The integrity of the RNA was assessed via gel electrophoresis, and the concentration was measured spectrophotometrically (Nanodrop Technologies, Montchanin, DE). One microgram of total RNA from all samples was reverse-transcribed simultaneously using the high-capacity cDNA Synthesis Kit according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). The quantitative PCR amplifications were performed using power SYBR green mix (Applied Biosystems) with a 7300 AB real-time PCR machine (Applied Biosystems). Primer pairs were selected for their specificity and efficiency: XBP1 (forward 5’-CCAGGAGTTAAGACCG-3’ and reverse 5’-CAACAGTGTCAGATCC-3’) and glyceraldehyde-3-phosphate dehydrogenase (forward 5’-GACCCCTTCATTGACCCTCAC-3’ and reverse 5’-CTTCTCCATGTTGTTGAAGA-3’); RAMP4 (forward 5’-CTGCCTAGAAGCTGAAGA-3’ and reverse 5’-TTCGAGGCGCAGAACA-3’), protein disulfide isomerase-P5 (Pdia5) (forward 5’-TGAAGTGATGACAAACCTG-3’ and reverse 5’-ATCCGG-GAAGTTTATCCCCCA-3’), HEDJ (forward 5’-AAAGAAAAGAGATGACAAGCA-GAAG-3’ and reverse 5’-TGGCTGATCACCCTTCAAAA-3’), and Znf25 (forward 5’-AGCGCGGACGAGTACGA-3’ and reverse 5’-TGTATGCTCAGATTTGGAAGGGTCT-3’); Erdj4 (forward 5’-CCGCGCAGGTTATTAGAAATGG-3’ and reverse 5’-GATG-GCTTGGAGGCCAG-3’), Ps8 (forward 5’-CTACGCTG-CAGTTGATG-3’ and reverse 5’-TGCTTGGCTGAACTCTTC-3’), and XBP1s (forward 5’-AAAGAAAAGAGATGACAAGCA-GAAG-3’ and reverse 5’-TGGCTGATCACCCTTCAAAA-3’). Primer sequences and target gene expression levels were determined by the comparative threshold cycle (dCT) method, and mRNA ratios were given by 2-ΔΔCt. PCR controls run in the absence of template were consistently negative.

NPS Glomerular Sample Preparation

For mice treated with NPS (generated by Lampire Biologic Laboratories, Ottsville, PA), experiments were performed as previously described in our laboratory.41 Mice were briefly preimmunized 3 days before administration of NPS via intraperitoneal injection of 250 μg of rabbit IgG (Jackson Immonoresearch Laboratories) in 250 μl of 1:1 emulsion with complete Freund adjuvant (Sigma-Aldrich).

GN was induced with 200 μl of NPS as previously described.41 Preimmune rabbit serum was used as a negative control. Mice were euthanized, kidneys were gathered, and the glomeruli were harvested by Percoll gradient, then processed for Western blot and for immunofluorescence.

Biochemical Measurements: Urine Albumin, Urine Creatinine, and Plasma Creatinine

Urine samples were collected from the Pod-Set63/ Xbp1 DKO and control littermates. Albuminuria was assessed qualitatively by 10% SDS-PAGE, followed by Coomassie blue staining. Urine albumin levels were measured qualitatively in duplicate using an albumin ELISA quantitation kit, according to the manufacturer’s protocol (Bethyl Laboratories, Inc, Montgomery, TX). The absorbance read at 450 nm (glomax multidetection system, Promega, Sunnyvale, CA) as previously described.41 Urine and plasma creatinine were measured in duplicates for each sample with an ELISA quantification kit (Bioassay Systems, Haywood, CA) at an absorbance of 490 nm (Microplate Reader; Bio-Rad, Japan).

Statistical Analyses

Urine electrolytes were analyzed by O’Brien Center Physiology Core at Yale University School of Medicine. The data are expressed as mean ± SEM. Statistical significance was determined with P<0.05, using Excel Software for Windows (Redmond, WA) using the t test.

Study Approval

All procedures were approved by Yale University IACUC and followed the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

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DISCLOSURES

None.

REFERENCES


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