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Urinary ERdj3 and mesencephalic astrocyte-derived neutrophic factor identify endoplasmic reticulum stress in glomerular disease

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Abstract
Podocyte injury and endoplasmic reticulum (ER) stress have been implicated in the pathogenesis of various glomerular diseases. ERdj3 (DNAJB11) and mesencephalic astrocyte-derived neurotrophic factor (MANF) are ER chaperones lacking the KDEL motif, and may be secreted extracellularly. Since podocytes reside in the urinary space, we examined if podocyte injury is associated with secretion of KDEL-free ER chaperones from these cells into the urine, and if chaperones in the urine reflect ER stress in glomerulonephritis. In cultured podocytes, ER stress increased ERdj3 and MANF intracellularly and in culture medium, whereas GRP94 (KDEL chaperone) increased only intracellularly. ERdj3 and MANF secretion was blocked by the secretory trafficking inhibitor, brefeldin A. Urinary ERdj3 and MANF increased in rats injected with tunicamycin (in the absence of proteinuria). After induction of passive Heymann nephritis (PHN) and puromycin aminonucleoside nephrosis (PAN), there was an increase in glomerular ER stress, and appearance of ERdj3 and MANF in the urine, coinciding with the onset of proteinuria. Rats with PHN were treated with the chemical chaperone, 4-phenyl butyrate (PBA), starting at the time of disease induction, or after disease was established. In both protocols, 4-PBA reduced proteinuria and urinary ER chaperone secretion, compared with PHN rats treated with saline (control). In conclusion, urinary ERdj3 and MANF reflect glomerular ER stress. 4-PBA protected against complement-mediated podocyte injury and the therapeutic response could be monitored by urinary ERdj3 and MANF.

Introduction

Glomerulopathies, such as membranous nephropathy (MN), focal segmental glomerulosclerosis (FSGS), diabetic nephropathy, and lupus nephritis, are accompanied by microenvironmental alterations and disturbances in protein homeostasis (proteostasis) in glomerular cells. As a result, cells can activate adaptive responses that increase their capacity to cope with stress, metabolic reprogramming, cell survival pathways, and communication networks with the immune system [1, 2]. Typically, adaptive responses occur early after the onset of stress, before the initiation of maladaptive repair processes and cell death [3]. Detection of adaptive responses constitutes an opportunity for the early diagnosis of ongoing tissue injury, which is crucial for the development of preventive and therapeutic strategies. In many glomerulopathies, the glomerular visceral epithelial cell (GEC) or podocyte is the target of injury. Podocytes have a complex morphology characterized by cell bodies with projecting interdigitating foot processes that are bridged by filtration slit diaphragms. These cells are metabolically robust, as they produce slit-diaphragm proteins (including nephrin), adhesion molecules, and glomerular basement membrane (GBM) components, and are critical to the maintenance of glomerular permselectivity [4].

During translation, secreted, lumenal, and membrane proteins, including the podocyte proteins noted above, are translocated into the endoplasmic reticulum (ER). Here, they are covalently modified to attain a correctly folded conformation by the action of folding enzymes and chaperones, prior to being transported to the Golgi pathway [5]. Maturation of proteins depends on appropriate levels of

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glucose, intracellular calcium, and redox environment. Disruption of ER function, due to factors such as ATP or ER calcium depletion, results in accumulation of misfolded proteins, ER stress, and activation of the unfolded protein response (UPR) [5–8]. There are three UPR pathways: activating transcription factor 6 (ATF6) is cleaved by proteases and the cleaved fragment enters the nucleus to induce transcription of genes encoding ER chaperones and components of ER-associated degradation. Inositol-requiring enzyme 1α (IRE1α), a protein kinase and RNase, splices X-box binding protein 1 (Xbp1) mRNA to yield a potent transcriptional activator of the above-mentioned genes. PKR-like ER kinase phosphorylates eukaryotic translation initiation factor 2α (eIF2α) to reduce translation and protein load on the damaged ER [8]. The UPR maintains ER proteostasis, facilitates recovery from stress, and may protect against further stresses (termed "adaptive" UPR), but sustained or prolonged ER stress may be cytotoxic.

Most chaperones residing in the ER, including BiP (GRP78) and GRP94, contain a C-terminal KDEL-ER retention sequence, which binds to the KDEL receptor. However, certain ER chaperones, which typically lack KDEL, are reported to be secreted from cells. Their secretory role(s) have not been defined conclusively, but secreted chaperones may be involved in maintaining extracellular proteostasis [9]. The ER HSP40 co-chaperone, ERdj3 (DNAJB11), has been identified as a secreted chaperone that is upregulated as part of the UPR [10]. ERdj3 binds to misfolded proteins in the ER, and delivers them to BiP to facilitate folding. During ER stress, when the BiP machinery is overwhelmed with misfolded proteins in the ER, ERdj3 remains bound to misfolded clients and accompanies them extracellularly to reduce their proteotoxic aggregation [10, 11]. Another ER chaperone, identified as being upregulated during the UPR and secreted, is mesencephalic astrocyte-derived neurotrophic factor (MANF or Armet). MANF was discovered as a dopaminergic neurotrophic factor in astrocyte-conditioned medium [12]. Although MANF lacks KDEL, it contains a C-terminal RTDL sequence that can potentially bind to KDEL receptors with lower affinity, compared with KDEL. Consequently, under nonstressed conditions, MANF is efficiently retained in the ER, and is not secreted, but during ER stress, KDEL chaperones could saturate binding to the KDEL receptors, allowing secretion of non-KDEL proteins, such as MANF [13]. Both ERdj3 and MANF are expressed in many cell types, including podocytes and other kidney cells [14].

There is evidence for ER stress in human glomerulopathies and their experimental counterparts [6, 11], including MN, or passive Heymann nephritis (PHN) in rats, and FSGS or puromycin aminonucleoside nephrosis (PAN) [15–17]. Idiopathic MN and FSGS are common causes of nephrotic syndrome in humans, and important causes of chronic kidney disease [18, 19]. The primary aim of treatment is to reduce proteinuria, but existing treatments involving immunosuppression are only partially effective and have significant toxicity, or treatments lack specificity [20]. In experimental and human MN, antibodies binding to podocyte antigens activate complement, leading to C5b-9-induced podocyte injury [18]. In PHN, complement C5b-9 induces an adaptive UPR, while in human MN, podocytes show a dilated and expanded ER [6] and increased ubiquitin content [21], in keeping with protein misfolding and ER stress. Podocyte-specific deletion of the UPR transducer IRE1α in mice exacerbated podocyte injury and proteinuria in anti-GBM nephritis, indicating that the UPR is protective in complement-induced podocyte injury [11, 22]. Human FSGS is likely caused by a circulating factor toxic to podocytes [19]. In PAN, upregulation of BiP in podocytes coincided with the development of proteinuria [16]. In addition, increased glomerular expression of ER stress markers, such as BiP or C/EBP homologous protein (CHOP), was shown in kidney biopsies of patients with MN and FSGS, as well as minimal change disease and proliferative glomerulonephritis [6, 23, 24].

Given the potentially important role of ER stress in glomerular disease, noninvasive biomarkers for detecting glomerular ER stress would be advantageous. Indeed, angiogenin, MANF and cysteine-rich with epidermal growth factor-like domain protein 2 (CRELD2) have been proposed as urinary glomerular or tubular ER stress biomarkers in experimental models of nephrotic syndrome or in human patients [25–27]. Since podocytes reside in the urinary space, we examined if podocyte injury is associated with secretion of KDEL-free ER chaperones from these cells into the urine, and if presence of the chaperones in the urine reflects ER stress in glomerulopathies. We show that excretion of the ER chaperones, ERdj3 and MANF, coincides with the onset of proteinuria and increased glomerular expression of ER chaperones in PHN and PAN. Treatment of PHN rats with the chemical chaperone, 4-phenyl butyric acid (4-PBA) to improve protein folding in the ER [28] led to a reduction in proteinuria, as well as a decrease in urinary ERdj3 and MANF.

Materials and methods

Materials

Cell culture reagents were purchased from Wisent Inc. (Saint-Jean-Baptiste, QC). Lipofectamine 2000 was from Invitrogen Life Technologies (Burlington, ON). Rabbit (SC-11402 RRID:AB_2119050) and rat anti-GRP94 antibodies (SC-32249 RRID:AB_627676) were from Santa Cruz Biotechnology. Rabbit anti-ERdj3 (15484-1-AP

RRID:AB_2094400) and anti-ATF4 antibodies (10835-1-AP RRID:AB_2058600) were from Proteintech (Rosemont, IL). Rabbit anti-MANF antibody (PAB13301 RRID: AB_10546841) was from Abnova (Walnut, CA). Rabbit anti-ERP57 (AD1-SPA-585F RRID:AB_10616507), rabbit anti-GRP78/BiP (SPA-826 RRID:AB_1193549), and rabbit anti-calnexin antibodies (SPA-860 RRID:AB_2069021) were from Enzo Life Sciences (Ann Arbor, MI). Rabbit anti-Wilm's tumor-1 (WT1) antibody C-19 (sc-192 RRID: AB_632611) was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit IgG (I8140), normal goat serum (G 9023), rabbit monoclonal anti-actin antibody (A2066 RRID: AB_476693), tunicamycin (TM), thapsigargin (TP), dithiothreitol (DTT), and puromycin aminonucleoside (PA; P7130) were from Sigma-Aldrich (St. Louis, MO). Rabbit anti-nephrin antiserum was kindly provided by Dr Tomoko Takano (McGill University, Montreal, QC) and was described previously [29, 30]. Brefeldin A (BFA) was from Cayman Chemical Co. (Ann Arbor, MI). ERdj3^{WT} (wild type) and ERdj3^{KDEL} cDNAs, subcloned in the pcDNA3.1 (+) vector [10], were kindly provided by R. Luke Wiseman (Scripps Research Institute, La Jolla, CA). The MANF–CD4-CD2 plasmid was from Addgene (RRID:Addgene_52026). The cDNA sequence is MANF (1–179 a.a), with CD4–CD2 fusion protein (179–366 a.a) and a hexahistidine tag epitope (6xHis) inserted downstream of RTDL at the C-terminus. Sodium phenyl butyrate (4-PBA, SPB 026/14–15) was from Scandinavian Formula, Inc. (Sellersville, PA, USA). Sheep anti-Fx1A antiserum was prepared as described previously [31]. Reactivity of ERdj3- and MANF-specific antibodies with their target proteins was previously validated by showing reductions in signal in shRNA-treated cells [10] or knockout (KO) mice [26].

Cell culture, transfection, and cytotoxicity assays

Primary cultures of GECs were established from explants of rat glomeruli, and were characterized previously [32]. Rat GECs were maintained in K1 medium [32]. Transfection experiments were carried out in COS-1 cells, which were cultured in DMEM supplemented with 10% FBS. Cells were utilized between passages 4 and 50. Cells were transiently transfected with Lipofectamine 2000 in OptiMEM according to the manufacturer's instructions. At 24 h post transfection, the transfection mix was replaced with fresh medium. Conditioned medium was harvested from the cells and centrifuged at 500 rpm for 7 min to remove any dead cells before processing for SDS-PAGE and immunoblotting. TM, TP, and BFA stock solutions were prepared in DMSO, and were added to cells in medium at final concentrations of 0.1–0.2%. In control incubations, DMSO alone (vehicle) was added to cells at the same concentration. Cell death/

viability was assessed using Hoechst H33342 and propidium iodide staining, or measuring release of lactate dehydrogenase (LDH) [33, 34]. Specific LDH release was calculated as $(S)/(S + T) \times 100$, where S represents the percentage of total LDH released into cell supernatants and T represents the percentage of LDH in the insoluble part of the cell.

Immunoblotting

GECs, COS-1 cells, and isolated rat glomeruli were lysed in buffer containing 1% Triton X-100 (lysis buffer) supplemented with 10 μl/ml protease inhibitor cocktail (BioShop) as described previously [34]. Protein concentration was measured with the Bradford assay. Samples were subjected to SDS-PAGE, and proteins were electrophoretically transferred to a polyvinylidene fluoride membrane of 0.2 μm pore size. After blocking, membranes were incubated with primary antibody overnight (4 °C), followed by horseradish peroxidase-conjugated secondary antibody for 1 h (22 °C). To examine secretion of ERdj3 into conditioned cell medium, equal volumes of media (60 μl) were loaded on SDS-PAGE. For detection of MANF secretion into the media, conditioned media were harvested from GECs and concentrated using Amicon Ultra Centrifugal filter units (10 kDa; Merck, Darmstadt, Germany), and equal volumes were loaded on SDS-PAGE. Urine samples were normalized to urine creatinine excretion, such that the urine volumes applied to each gel lane reflected the same amount of urine creatinine (usually 5 μg). Density of specific bands was measured using ImageJ software and the density of bands in lysates was normalized to actin. Although there was a wide range in the intensity of certain signals, we took precautions to perform quantification safeguarding the linearity of signals.

Experiments in rats and mice

Male Sprague-Dawley rats (150–250 g, Charles River, St. Constant, Quebec) were used in the in vivo experiments. Rats were injected intraperitoneally with TM (1 mg/kg) dissolved in DMSO at 2 mg/ml and diluted in sterile PBS. Urine was collected for 3–4 h in metabolic cages. Rats were euthanized 24–72 h after injection. Glomeruli were isolated by differential sieving [31]. PHN was induced in rats by a single intravenous injection of 0.4 ml of sheep anti-rat Fx1A antiserum, as described previously [31, 35]. Alternatively, two injections of 0.2 ml were administered on 2 consecutive days. This protocol resulted in the induction of proteinuria within 5–7 days of injection. PAN was induced by a single intravenous injection of PA (80 mg/kg) [16]. Urine was collected as above. Rats were euthanized after completion of urine collections, and

kidneys were harvested for immunofluorescence staining, as well as for isolation of glomerular and tubular fractions [31]. 4-PBA was administered at 1 g/kg/day in the drinking water [36]. The 4-PBA dosage was adjusted in fresh drinking water every 3 days based on the weight of the rats and the amount of water consumed. In the control group, NaCl was added to the drinking water so that the Na concentrations in all groups of rats were similar. Mice with cystinosis (KO of the CTNS gene which encodes cystinosin) were characterized previously [37, 38]. Urine from these mice was kindly provided by Dr Paul Goodyer and Emma Brasell (McGill University). Studies were carried out in accordance with guidelines established by the Canadian Council on Animal Care, and the animal protocol was approved by the McGill University Animal Care Committee.

Rat urine protein and creatinine measurements

Collected urine samples were kept on ice, and were centrifuged immediately post collection ($1800 \times g$ for 10 min) to remove cell debris, and then stored at $-80\,^{\circ}\mathrm{C}$. Urine protein was measured using the Bradford assay. Urinary creatinine was measured using a kit that employs a picric acid-based method (Cayman Chemical Co.). Samples were analyzed in duplicate. Using the standard curve equation of best fit, creatinine concentrations were obtained and the protein/creatinine ratios were calculated in μg/μg for each animal.

Immunofluorescence staining

Isolation, embedding, and cryosectioning (4-μm sections) of renal cortex were described previously [34]. Frozen cryosectioned slides were air dried for 30 min at 22 °C and fixed in 4% paraformaldehyde for 15 min at 22 °C. Slides were blocked in 10% normal goat serum in PBS, for 1 h at 22 °C [34]. Sections were incubated with primary antibody (anti-ERdj3, anti-MANF, anti-BiP, or anti-Fx1A) or nonimmune IgG or serum (negative control) overnight at 4 °C, followed by secondary antibody for 1 h at 22 °C [34]. For immunofluorescence staining with anti-Fx1A, after incubation with secondary antibody, tissues were incubated with 1 μg/ml of Hoechst H33342 (nuclear stain) for 15 min at 22 °C and then mounted. WT1 immunostaining was described previously [22]. Stained kidney sections were examined with a Zeiss Axio Observer fluorescence microscope with visual output connected to an AxioCam MRm monochrome camera. To allow comparisons of fluorescence intensities, all images were taken at the same exposure, with the length of exposure set to avoid camera saturation. Fluorescence intensity was quantified using ImageJ software [34].

Statistics

Data are presented as the mean ± standard error. Densitometry and fluorescence quantification are presented in arbitrary units (percent of maximum value in the data set). One-tail t-tests were employed to assess differences between two groups. For more than two groups, one-way and two-way analysis of variance was employed. Post hoc comparisons and adjustments of the critical p value were conducted according to the Bonferroni method. Graphs were produced using Microsoft Excel.

Results

ER stress induces ERdj3 secretion in GECs

Rat GECs were treated with TM, which activates ER stress by blocking N-linked glycosylation in the ER [39], or TP, which inhibits the ER calcium ATPase, thereby blocking ER calcium uptake and depleting ER calcium [40]. By immunoblotting of lysates and conditioned media, basal intracellular, and secreted ERdj3 (chaperone that lacks the KDEL motif) was evident at 3, 6 or 24 h (Supplementary Fig. 1A). TM and TP increased intracellular ERdj3, as well as KDEL chaperones (GRP94, ERP57, and/or BiP) at 6 and/or 24 h (Supplementary Fig. 1A and Fig. 1). TM induces expression of an aglycosylated form of ERdj3 (at 37 kDa; Supplementary Fig. 1A and Fig. 1a, band B), while mature glycosylated ERdj3 migrates at 41 kDa (band A). TM or TP stimulated extracellular secretion of ERdj3 (37 and 41 kDa forms, respectively) at 24 h, although changes prior to 24 h were not consistent (Supplementary Fig. 1A and Fig. 1). KDEL chaperones were not, however, detected extracellularly (Fig. 1). There was no change in the intracellular expression of calnexin, and no secretion of this chaperone (Fig. 1). Similarly to TP, DTT (a reducing agent that prevents formation of disulfide bonds between cysteine residues in proteins) enhanced intracellular expression and secretion of the 41 kDa ERdj3 (Supplementary Fig. 1B).

By analogy to GECs, TM stimulated secretion of aglycosylated ERdj3 (but not GRP94) in COS-1 cells, and increased intracellular ERdj3 and GRP94 (Supplementary Fig. 2A). Thus, secretion of ERdj3 was not restricted to GECs. Co-treatment with TM and the secretory trafficking inhibitor BFA completely blocked ERdj3 secretion, suggesting that ERdj3 secretion is through the canonical ER-Golgi pathway (Supplementary Fig. 2A). To examine if lack of the KDEL motif is responsible for ERdj3 secretion, COS-1 cells were transfected with ERdj3^{WT} or ERdj3^{KDEL} (ERdj3 fused with C-terminal KDEL). At a lower amount of plasmid DNA (0.1 μg), overexpression of ERdj3^{WT} increased extracellular ERdj3, while only slightly increasing

A
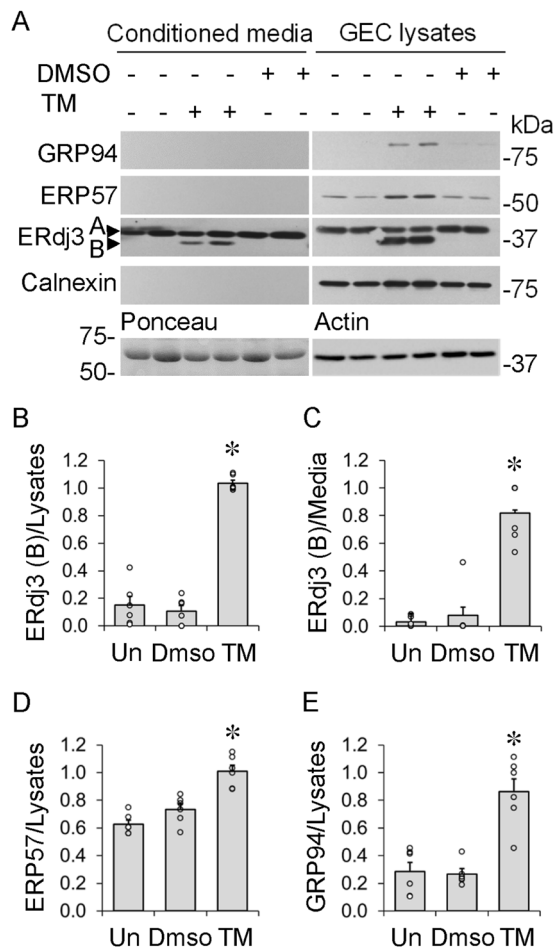
Fig. 1 ER stress induces ERdj3 secretion in GECs. Cells were untreated (Un) or treated with DMSO (vehicle) or TM (10 µg/ml) for 24 h. Lysates and conditioned media (60 µl) were immunoblotted as indicated. The upper ERdj3 band (band A) is the mature form, while the lower band (band B) is the aglycosylated form. Representative immunoblots and Ponceau stain (**a**) and densitometric quantification ((**b**)–(**e**); for ERdj3, the aglycosylated form was quantified). **b** *$p = 3 \times 10^{-8}$ untreated vs TM and $p = 4 \times 10^{-7}$ DMSO vs TM. **c** *$p = 3 \times 10^{-6}$ untreated vs TM and $p = 0.0002$ DMSO vs TM. **d** *$p = 6 \times 10^{-5}$ untreated vs TM and $p = 4 \times 10^{-5}$ DMSO vs TM. **e** *$p = 2 \times 10^{-7}$ untreated vs TM and $p = 5 \times 10^{-7}$ DMSO vs TM. $N = 4$ experiments performed in duplicate. The prominent band in the Ponceau stained membrane at ~67 kDa was identified as albumin.

intracellular ERdj3, compared with vector (Supplementary Fig. 2B). In contrast, overexpression of ERdj3^{KDEL} showed an exclusive increase of intracellular ERdj3. Transfection of COS-1 cells with a greater amount of plasmid DNA (0.5 µg) markedly increased extracellular ERdj3^{WT} (both immature and mature forms of ERdj3 were secreted); there was a smaller increase in intracellular ERdj3. At the higher dose of plasmid DNA transfection, a small amount of ERdj3^{KDEL} appeared in the medium (Supplementary Fig. 2B). Most likely, the high amount of ERdj3^{KDEL} saturated the KDEL receptors, and some of the protein escaped from the cells. BFA substantially inhibited secretion of endogenous and

ectopic ERdj3^{WT}, consistent with secretion through the canonical pathway (Supplementary Fig. 2C). Ectopic ERdj3^{WT} appeared as both mature and aglycosylated forms intracellularly and extracellularly (Supplementary Fig. 2B and C), implying that excess expression most likely overwhelmed the glycosylating capacity of the cells, and confirming that the aglycosylated form can be secreted.

ER stress induces MANF secretion in GECs

MANF is another ER chaperone that lacks the KDEL motif. In TM-treated GECs, MANF expression was upregulated both in cell lysates and concentrated conditioned medium (Fig. 2a–c), while GRP94 increased intracellularly (Fig. 2a and d), and not extracellularly (as shown in Fig. 1a). Although MANF does not contain KDEL, the four C-terminal amino acids in MANF are RTDL, which could potentially interact with the KDEL receptor with weaker affinity. To test if RTDL could modulate MANF secretion, COS-1 cells were transiently transfected with a cDNA encoding a 37 kDa His-tagged MANF–CD4–CD2 fusion protein (CD4–CD2 were inserted 3′ of RTDL). Addition of the CD4–CD2 sequence C-terminally to RTDL was reported to interfere with the function of RTDL [41]. Cells transfected with MANF–CD4–CD2 were treated with or without TM. Abundant ectopic MANF–CD4–CD2 was detected in unconcentrated medium, and TM had no significant effect on MANF–CD4–CD2 secretion (Supplementary Fig. 3A; MANF^E). Endogenous MANF is amply expressed in cell lysates, but is absent in unconcentrated medium (Supplementary Fig. 3A). This result implies that RTDL imparts at least a partial KDEL effect and reduces MANF secretion. BFA markedly reduced extracellular secretion of ectopic MANF–CD4–CD2, suggesting secretion through the canonical ER-Golgi secretory pathway (Supplementary Fig. 3B).

Finally, we performed experiments to exclude the possibility that TM-induced ER stress resulted in cell death, and that consequently the release of ERdj3 and MANF extracellularly was due to leakage of soluble intracellular proteins across damaged membranes. GECs were incubated with TM or DMSO (vehicle). At 24 h, cell death was trivial, i.e., viable cells were 93.8 ± 1.3% of total in TM and 95.4 ± 0.1% in the DMSO group. Apoptotic cells (fragmented nuclei but propidium iodide negative) were 3.8 ± 0.4% in TM and 4.0 ± 0.8% in DMSO. TM treatment resulted in a slightly greater late apoptotic or necrotic cells (propidium iodide-positive cells; 2.4 ± 0.9% in TM, compared with 0.6 ± 0.4% in DMSO); however, this slight increase was not confirmed by measuring release of LDH (3.9 ± 0.4% in TM and 2.8 ± 0.7% in DMSO). Therefore, TM-induced ERdj3 and MANF release into culture medium was not a consequence of cell death and membrane permeabilization.

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B C D
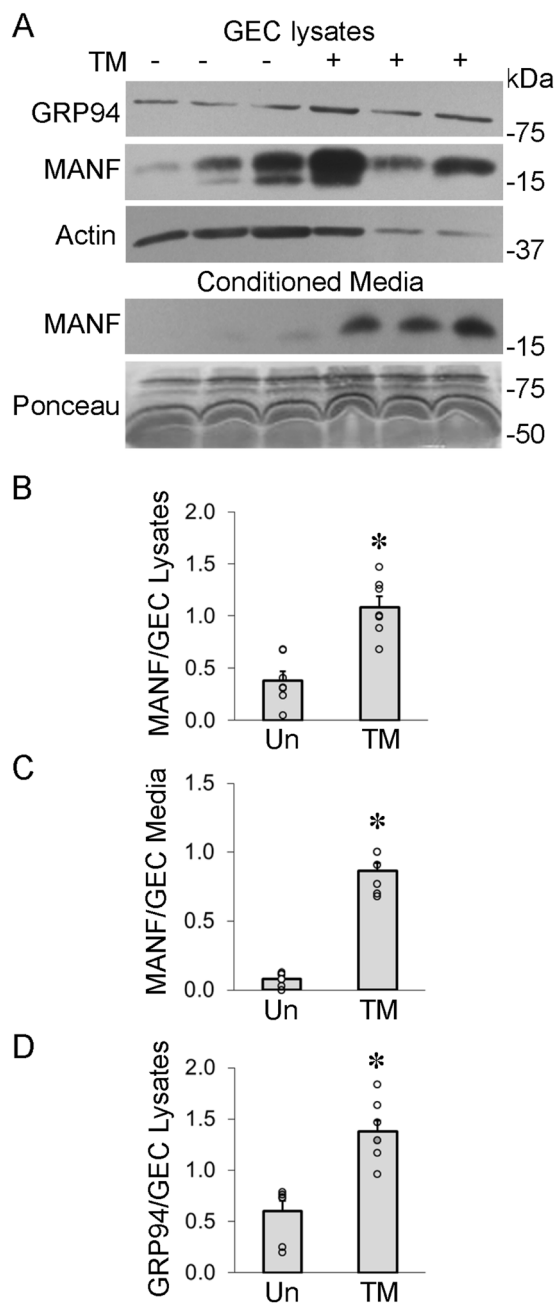
Fig. 2 ER stress induces MANF secretion in GECs. GECs were untreated (Un) or treated with TM (10 μg/ml) for 24 h. Conditioned media were concentrated tenfold prior to immunoblotting. Representative immunoblot and Ponceau stain (**a**) and densitometric quantification (**b**)–(**d**). **a** The lower MANF band is probably a degradation product. **b** $*p = 0.0001$, **c** $*p = 1 \times 10^{-8}$, and **d** $*p = 0.0001$ untreated vs TM. $N = 3$ experiments performed in duplicate or triplicate.

TM induces urinary excretion of ERdj3 and MANF in rats

The results described above show that in cultured GECs, ERdj3 and MANF both can be secreted extracellularly during ER stress. We proceeded to investigate if ERdj3 and

MANF are produced in ER stressed glomeruli and released into the urine in vivo. Initially, we induced ER stress in rats by injecting TM systemically. ERdj3 immunoreactivity (41 kDa) was present in the urine prior and post TM injection; however, the 37 kDa aglycosylated form appeared at 24–48 h post TM, but was absent in control (Supplementary Fig. 4A, B). MANF was not detected in the urine prior to TM injection, but appeared within 24 h and persisted at 48 h (Supplementary Fig. 4A, C). TM also increased aglycosylated ERdj3 and MANF in isolated rat glomeruli (Supplementary Fig. 4D–G). There were no consistent differences in the urine protein/creatinine ratios between control and TM-injected rats indicating that appearance of ERdj3 and MANF in the urine was independent of proteinuria (Supplementary Fig. 4).

Nephrin is an important podocyte-resident protein that undergoes post-translational modification in the ER and oligosaccharide processing in the Golgi, prior to being exported to the cell surface [42]. By immunoblotting, nephrin appears as a fully glycosylated mature form (~180 kDa), as well as an immature, ER luminal form (~170 kDa) [30]. Systemic administration of TM did not produce detectable changes in the ratio of mature to immature nephrin, nor in the total amount of mature nephrin after 24 h (Supplementary Fig. 4H). However, TM induced the appearance of a third faster migrating band (~140 kDa), which most likely represents an aglycosylated form of nephrin [30]. This result supports the view that TM impaired ER function and induced ER stress in podocytes.

To determine if TM induced tubular injury in these rats, urine samples from the five TM-injected rats were tested for glycosuria using glucose-sensitive strips and were compared with four controls. TM did not induce any detectable glycosuria. Second, rat kidney sections were incubated with sheep anti-Fx1A antiserum, followed by fluorescein-anti-sheep IgG, to visualize the integrity of the tubular brush border [43]. There were no significant differences in the pattern or intensity of brush border staining between TM-treated and control rats, implying that TM did not damage brush border proteins (Supplementary Fig. 4I).

Glomerular proteostasis is disrupted in PHN

PHN, a model of complement-induced podocyte injury, is associated with glomerular ER stress [16, 35]. To establish PHN, rats were injected with anti-Fx1A antiserum. Urine samples, collected pre injection and up to day 14 post injection, showed onset of significant proteinuria on day 7, which increased further by day 14 (Fig. 3a). The urine protein consisted almost exclusively of albumin (Supplementary Fig. 5A). Rats with PHN had larger glomeruli, compared with control (~35% greater glomerular area), and the number of podocytes per glomerular area (WT1-positive
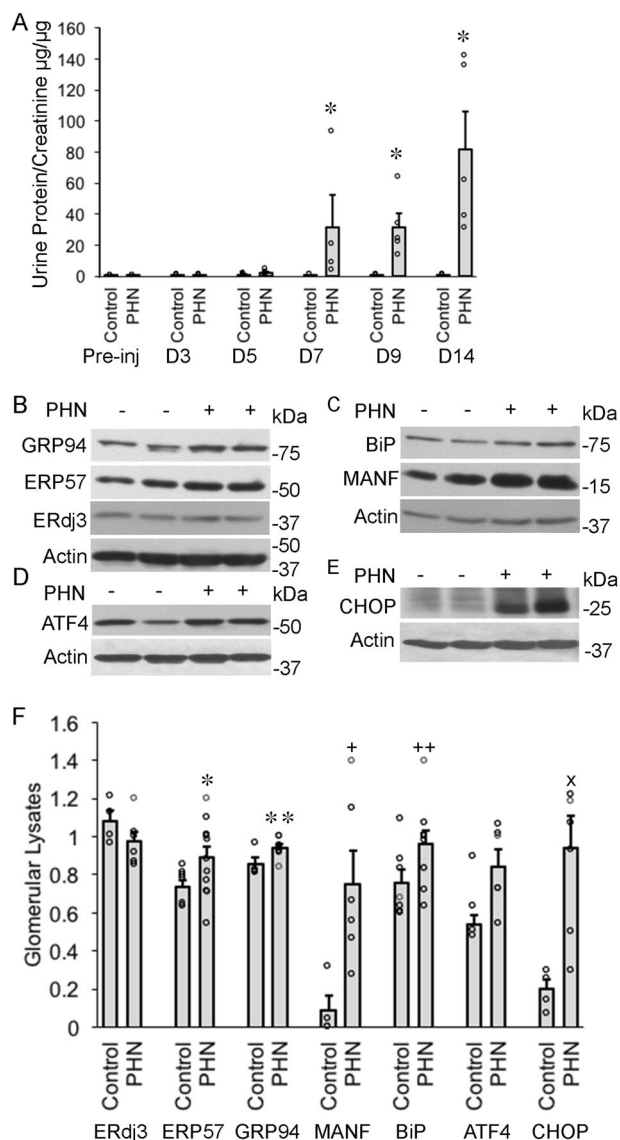
Fig. 3 Glomerular ER stress is evident in PHN. PHN was induced by intravenous injection of sheep anti-Fx1A antiserum. **a** Urine protein/creatinine ratio increased significantly on days (D) 7–14 (Pre inj pre injection). *$p = 0.006$ Control ($N = 4$ rats) vs PHN ($N = 6$ rats). **b–f** Glomeruli were isolated on day 17, and lysates were immunoblotted, as indicated. **b–d** Representative immunoblots (ERdj3 is the glycosylated form). **f** Densitometric quantification. ERP57 *$p = 0.03$, GRP94 **$p = 0.02$, MANF +$p = 0.001$, BiP ++$p = 0.04$, ATF4 $p = 0.06$, CHOP x$p = 0.005$ control vs PHN.

cells) was reduced significantly in PHN (by 33%; Supplementary Fig. 5B, C), in keeping with podocyte injury.

To verify that ER stress was induced in PHN, glomeruli were isolated after day 14, and lysates were immunoblotted for ER chaperones and other mediators of ER stress. Compared with controls, rats with PHN showed significantly increased glomerular expression of ERP57, GRP94, BiP, and MANF, although ERdj3 did not change significantly (Fig. 3b–f). ATF4 expression tended to be greater in PHN, while its downstream target, CHOP,

increased significantly. These results are keeping with enhanced glomerular ER protein misfolding in PHN.

## Urinary excretion of ERdj3 and MANF is increased in PHN

Changes in glomerular ER stress parameters most likely originate in podocytes, the primary site of complement-mediated injury in PHN [20]. Since podocytes reside in the urinary space, we asked if the secretion of ERdj3 and MANF from podocytes into the urine can serve as a biomarker of podocyte ER stress in PHN. ERdj3 and MANF immunoreactivity was not detected consistently in urine of rats with PHN on days 3 and 5, but was significantly greater in PHN urine on days 7, 9 and 14, compared with control (Fig. 4a–d), thus coinciding with development of proteinuria. GRP94 was not detected in PHN or control rat urine (result not shown). We then examined if the level of proteinuria correlates with the amount of urinary ERdj3 and MANF excretion on days 7, 9 and 14 in individual rats with PHN. The correlation was only moderate for ERdj3, and very weak for MANF (Supplementary Fig. 6A, B). Furthermore, while the mean levels of both ERdj3 and MANF excretion were increased in PHN, the correlation between ERdj3 and MANF excretion in individual proteinuric rats was weak (Supplementary Fig. 6C).

Urinary MANF and ERdj3 detect podocyte ER stress in rats with PAN

Glomerular ER stress has been demonstrated in PAN, a model of toxin-induced podocyte injury [16, 17]. Accordingly, we examined if urinary ERdj3 and MANF can be used to identify podocyte ER stress. Rats with PAN developed proteinuria on day 5, which reached a maximum on day 10 and declined thereafter (Fig. 5a). In this model of PAN, light microscopy of kidney sections showed normal glomeruli, without sclerosis, GBM thickening or infiltrating inflammatory cells (not shown). In isolated rat glomeruli, immunoblotting revealed increased expression of ERP57, CHOP, and ERdj3 in PAN, although there was not a significant increase in MANF (Fig. 5b–d). Compared with control, both ERdj3 and MANF were significantly increased in the urine of PAN rats as early as day 5 post injection of PA and reaching a maximum on day 10 (Fig. 5e–h).

4-PBA reduces proteinuria and urinary chaperone excretion in PHN

The above studies show that in PHN, complement-mediated podocyte injury is associated with ER stress, and have identified two urinary ER chaperones that reflect glomerular ER stress. The aim of the next set of experiments was to
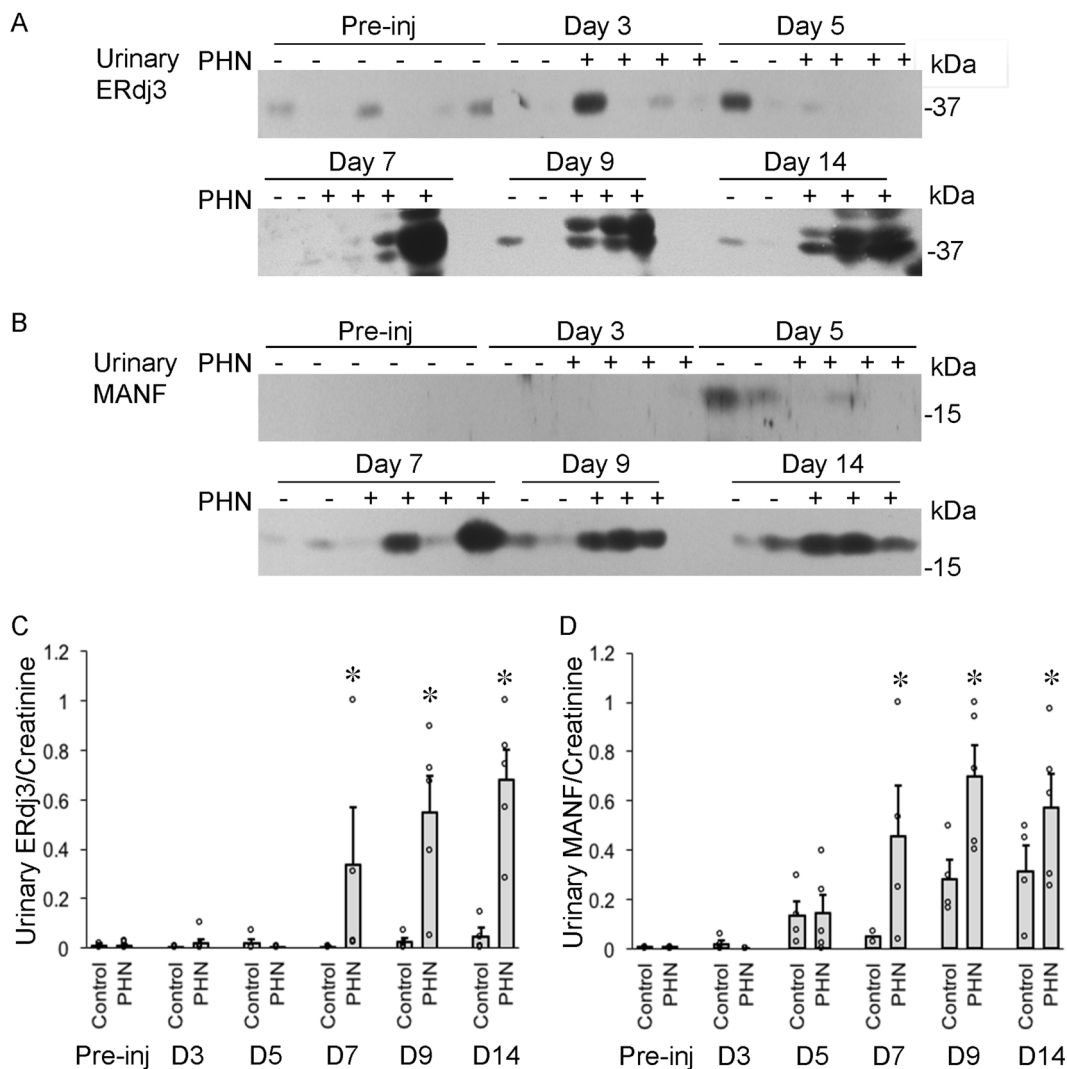
Fig. 4 Urinary ERdj3 and MANF excretion in PHN. Urine samples were collected pre injection (Pre inj), and on days (D) 3–14 from rats with PHN and control rats. Urine samples were loaded on gels such that each lane contains an equal amount of creatinine (5 µg/lane). **a, b** Representative immunoblots. In some urine samples, glycosylated ERdj3 appeared as a doublet; the lower band may be a degradation product. **c, d** Densitometric quantification of urinary ERdj3. **c** *$p = 0.0001$ control vs PHN and MANF. **d** *$p = 0.002$ control vs PHN. There are 2–4 control rats and 4–5 PHN rats per time point.

determine if 4-PBA can ameliorate podocyte injury in PHN by improving protein folding in the ER and reducing ER stress, and if the reduction of ER stress by 4-PBA can be monitored using urinary ERdj3 and MANF. Four groups of rats were studied, including control, PHN rats drinking saline, and PHN rats drinking 4-PBA, starting treatment the same day as the anti-Fx1A injection (day 0) or on day 7. Urine samples were collected pre injection, and on days 5–23 post injection. PHN rats drinking saline demonstrated a significant increase in the mean protein/creatinine ratio on days 7–23 (Fig. 6). 4-PBA treatment of PHN rats starting on the day of antibody injection significantly reduced the protein/creatinine ratio on days 5–23 (by $32 \pm 10\%$), compared with PHN rats drinking saline (Fig. 6a). Similarly,

treatment of PHN rats starting on day 7 after injection significantly reduced protein/creatinine ratio on days 9–23 (by $42 \pm 8\%$), compared with the saline group (Fig. 6b). Glomeruli were isolated at the end of urine collections, and lysates were subjected to immunoblotting. A significant increase in glomerular expression of GRP94 was observed in PHN rats on saline vs control (Supplementary Fig. 7). 4-PBA tended to reduce glomerular GRP94, although the reduction did not reach statistical significance.

We then examined if 4-PBA reduces urinary excretion of ERdj3 and MANF. Prior to the induction of PHN, urinary levels of ERdj3 and MANF were low or undetectable. Comparably low levels persisted in control rats. Urinary ERdj3 and MANF both increased in PHN rats drinking
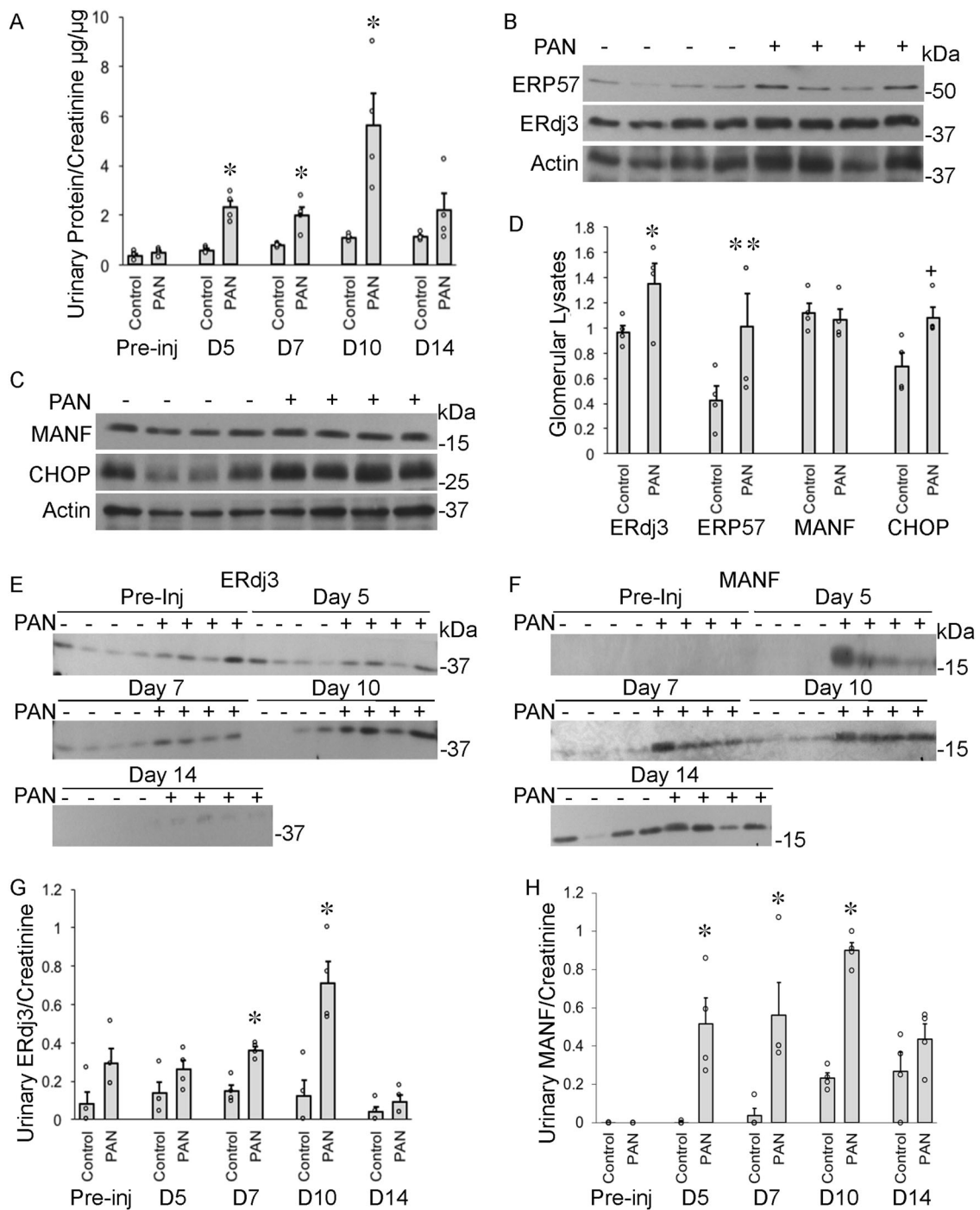
Fig. 5 Urinary ERdj3 and MANF in PAN. a Urine protein/creatinine ratio increased significantly on days (D) 5–10 after administration of PA. $*p = 0.0009$ control vs PAN rats (four rats per group). **b, c** Glomeruli were isolated on day 15, and lysates were immunoblotted, as indicated. **b, c** Representative immunoblots. **d** Densitometric quantification. ERdj3 (glycosylated) $*p = 0.04$, ERP57 $**p = 0.04$, MANF $p = 0.30$. CHOP $+p = 0.01$ control vs PAN. **e–h** Urine samples (5 μg of creatinine/lane) were immunoblotted with anti-ERdj3 or anti-MANF antibodies. **g** Densitometric quantification of glycosylated ERdj3. $*p = 0.005$ control vs PAN. **h** Densitometric quantification of MANF. $*p = 0.0001$ control vs PAN. There are four control rats and four PAN rats per time point.

saline (Fig. 7a–f). Similar to the effect on proteinuria, 4-PBA significantly reduced urinary ERdj3 excretion on days 5–14 when started on day 0 (by $46 \pm 16\%$) or day 7 (by 33–66%), compared with PHN rats drinking saline

(Fig. 7a, c, e). By analogy, 4-PBA reduced urinary MANF excretion when started on day 0 (by $88 \pm 8\%$), although the reduction did not reach statistical significance when 4-PBA was started on day 7 (Fig. 7b, d, f).
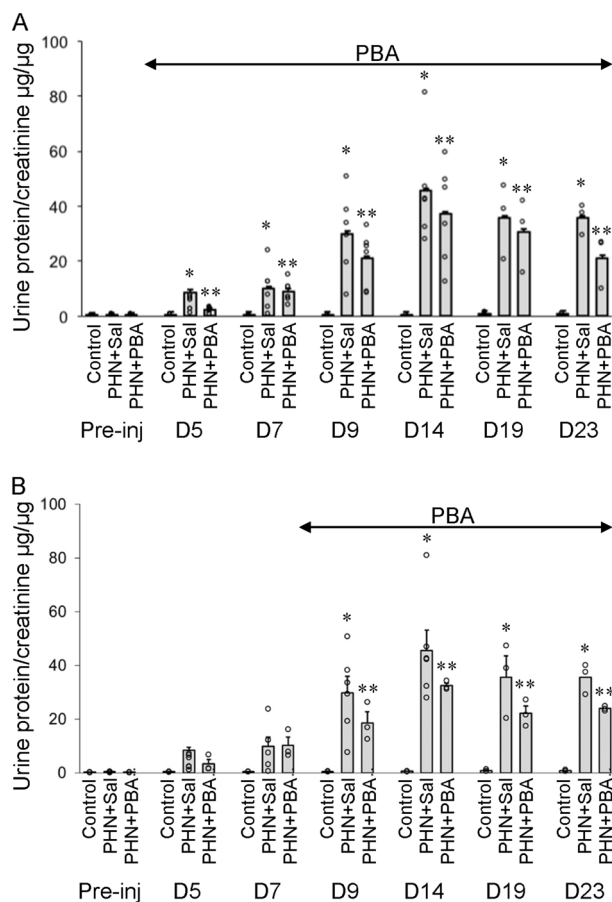
Fig. 6 4-PBA ameliorates proteinuria in PHN. a, b Urine protein/creatinine ratios in control rats ($N = 3$ rats), PHN rats treated with saline (Sal) in the drinking water ($N = 6$ rats), PHN rats treated with 4-PBA in the drinking water starting on the same day as the anti-Fx1A injection ($N = 6$ rats), and PHN rats treated with 4-PBA in the drinking water starting on day (D) seven after injection ($N = 3$ rats). **a** *$p = 2 \times 10^{-9}$ PHN + saline vs control, and **$p = 0.002$ PHN + 4-PBA vs saline. **b** **$p = 7 \times 10^{-5}$ PHN + 4-PBA vs PHN + saline.

Tubular ER stress and urinary ER chaperone excretion

Although injury in PHN originates in glomerular podocytes and the disease demonstrates glomerular ER stress [16, 35], we examined if ER stress is also evident in tubular cells. By immunoblotting of isolated kidney tubular fractions, there were no significant differences in tubular ERdj3 and MANF between PHN and control (Supplementary Fig. 8A). By immunofluorescence microscopy tubular MANF was not significantly different between PHN and control, while ERdj3 showed some increase in tubules (Supplementary Fig. 8B–G). Compared with control, the KDEL chaperones, GRP94 and ERP57, were increased significantly in PHN tubules (immunoblotting; Supplementary Fig. 8A). However, BiP was not increased in tubules (immunofluorescence microscopy; Supplementary Fig. 8H–J). In summary, using two techniques, we did not observe an increase in tubular

MANF in PHN, despite increases in the glomerulus. In PHN, both techniques showed that KDEL chaperones increased in the glomerulus, but increases in tubules were not consistent.

In the next series of experiments, we further examined if tubular ER stress can lead to urinary excretion of ER chaperones, and if tubular ER stress or urinary ER chaperones could result from proteinuria. We immunoblotted the urine samples of rats with PHN (day 14) and PAN (day 10) on the same membrane with anti-ERdj3 and anti-MANF antibodies. Densitometric quantification of the urinary ERdj3 and MANF signals showed that comparable levels of ERdj3 and MANF were excreted in the urine of rats with PHN and PAN even though PHN rats had ~15-fold greater mean urinary protein/creatinine (Supplementary Table 1). This result would argue against proteinuria being a major stimulus for secretion of chaperones from tubular cells into the urine. We then measured excretion of ERdj3 in urine of mice with experimental cystinosis, i.e., KO of the CTNS gene which encodes cystinosin. These mice develop a proximal tubulopathy and tubular proteinuria without significant podocyte injury, and they show ER stress [37, 38]. Specifically, GRP94 and BiP expression is increased in kidney lysates from CTNS KO mice, compared with wild type animals, implying ER stress in the tubular compartment due to lysosomal accumulation of cysteine crystals [38]. Moreover, CTNS KO embryonic fibroblasts show upregulation of the UPR and expansion of the ER [38]. Immunoblotting of urine samples from CTNS KO mice in the C57BL/6 background and from outbred CTNS KO mice did not show any consistent increases in ERdj3, compared with littermate controls (Supplementary Fig. 9).

Discussion

Aberrant ER proteostasis and ER dysfunction are associated with the initiation or development of a variety of glomerular diseases; however, methods to monitor ER stress using noninvasive biomarkers have been lacking. In the present study, we demonstrate that KDEL-deficient ER chaperones, ERdj3 and MANF, can potentially serve as biomarkers of ER stress. These chaperones can be measured in urine in experimental models of podocyte ER stress, including PHN and PAN. Moreover, in PHN, the chemical chaperone, 4-PBA, reduced proteinuria and urinary ERdj3 and MANF excretion.

We showed that both ERdj3 and MANF are secreted chaperones in cultured GECs, consistent with observations in other cell types [10, 44, 45]. TM induced an aglycosylated ERdj3, in keeping with earlier reports [46, 47]. Interestingly, aglycosylated ERdj3 was secreted from cells, and secretion of both glycosylated and aglycosylated ERdj3
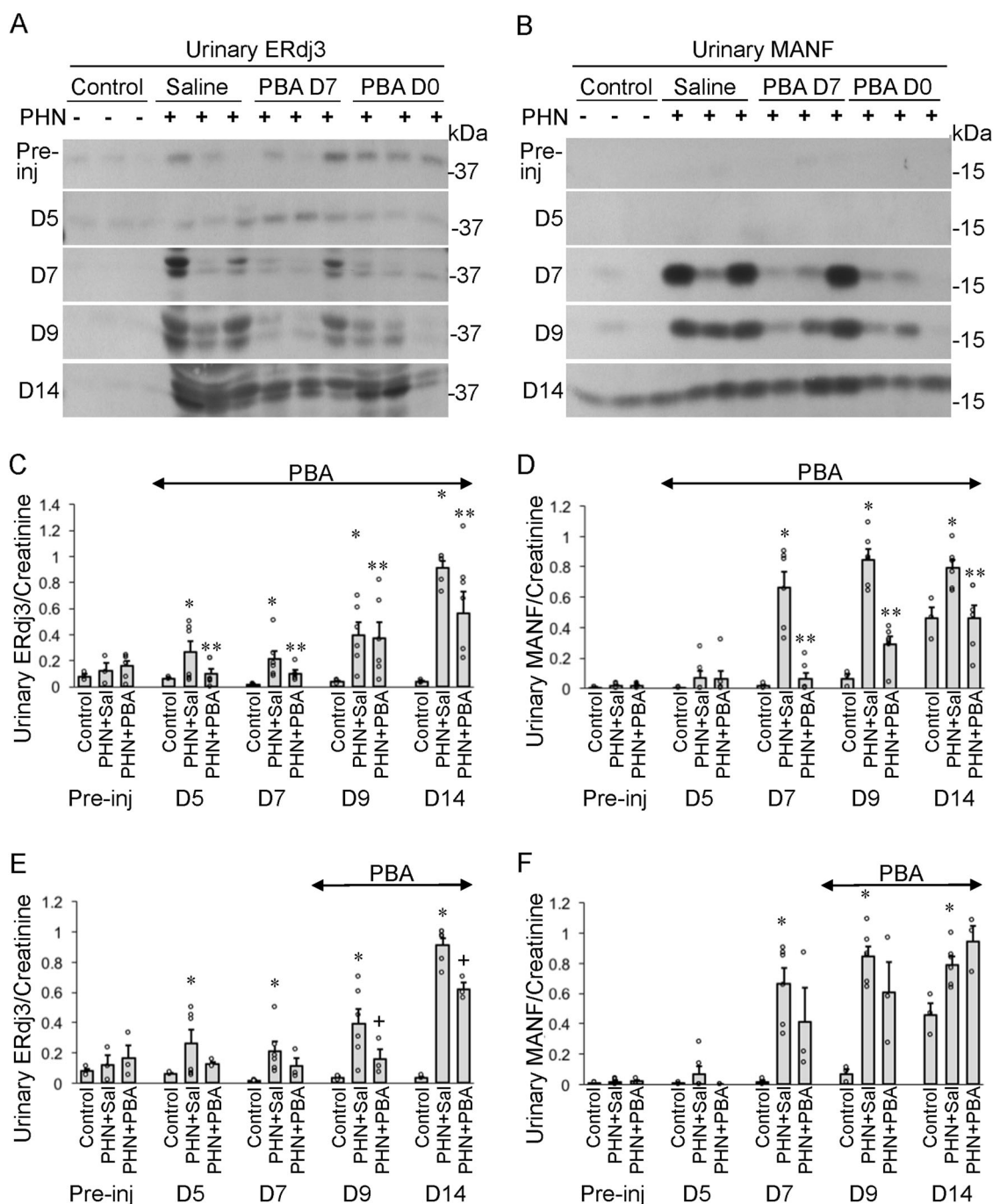
Fig. 7 4-PBA attenuates urinary excretion of ERdj3 and MANF in PHN. a, b Urine samples (5 μg of creatinine/lane) were immunoblotted with antibodies to ERdj3 or MANF. **c, e** Densitometric quantification of urinary ERdj3. $*p = 4 \times 10^{-6}$ PHN + saline vs control, $**p = 0.004$ PHN + saline vs 4-PBA starting on day (D) 0 until day 14, $^+p = 0.0005$ PHN + saline vs 4-PBA starting on day 7 until day 14. **d, f** Densitometric quantification of urinary MANF. $*p = 5 \times 10^{-7}$ PHN + saline vs control, $**p = 1 \times 10^{-5}$ PHN + saline vs 4-PBA starting on day 0 until day 14, $p = 0.14$ PHN + saline vs 4-PBA starting on day 7 until day 14. The number of animals is the same as in the legend to Fig. 6.

was inhibited with BFA, suggesting that both forms undergo secretion through the ER-Golgi pathway. In contrast, TP and DTT (which induce ER stress without affecting glycosylation of proteins) stimulated extracellular secretion of glycosylated ERdj3. As expected, fusion of the KDEL motif to the C-terminus of ERdj3 reduced its secretion. TM induced a robust intracellular increase of MANF and more modest secretion into the medium, in keeping with a previous study [45]. Attenuation of MANF secretion may also be due to its C-terminal RTDL motif,

which can potentially interact with the KDEL receptor [48]. Thus, competition for KDEL receptor binding by other chaperones may be required to increase MANF secretion.

In vivo, podocytes reside in the urinary space. Having established that ERdj3 and MANF are secreted upon induction of ER stress in cultured GECs, we examined if ER stress associated with podocyte injury in vivo would lead to secretion of these chaperones into the urine. Injection of rats with TM-induced ER dysfunction in podocytes (reflected by impaired nephrin glycosylation), increased glomerular expression of ERdj3 and MANF, and increased urinary excretion of the two chaperones, independently of proteinuria or proximal tubular renal cell injury. Next, the present study expanded earlier observations in PHN (complement C5b-9-mediated podocyte injury) and PAN (toxin-induced podocyte injury) by showing significant increases in the expression of glomerular KDEL-ER chaperones and CHOP [16, 17]. Generally, CHOP is believed to be a proapoptotic protein, but overexpression of CHOP in cultured GECs did not induce apoptosis [23]. In the context of intracellular ER stress, there was robust excretion of ERdj3 and MANF into the urine in both PHN and PAN, concurrent with onset of proteinuria. Increases in glomerular expression of ERdj3 and MANF were less consistent, suggesting that during ER stress, these chaperones are secreted rapidly from cells and may not accumulate intracellularly. In individual rats with PHN, the magnitude of urinary ERdj3 excretion correlated only weakly with MANF. This finding highlights the need for measuring two biomarkers to establish a diagnosis of glomerular ER stress. In previous studies, urinary MANF and another KDEL-free chaperone, CRELD2, were increased in a mouse model of nephrotic syndrome in which podocyte ER stress is activated by transgenic expression of the C321R laminin mutant in podocytes [26, 27]. Interestingly, in this study, urinary MANF and CRELD2 migrated at a much higher or lower molecular mass, respectively, on SDS-PAGE, compared with the usual molecular mass of MANF and CRELD2. The authors suggested that perhaps MANF and CRELD2 underwent dimerization or post-translational modification in the mouse urine [26, 27]. In our study, urinary MANF migrated at its usual molecular mass.

Chemical chaperones, such as 4-PBA, provide a more favorable environment in which a protein can fold, by increasing the strength of intramolecular hydrophobic bonds that are buried within a protein or lowering a protein's free energy state [49]. This may enable proper trafficking of membrane and secreted proteins, and/or prevent the proteotoxic effects of misfolded proteins. Previous studies have shown that 4-PBA attenuates ER stress in various experimental kidney diseases, including diabetic nephropathy and proteinuria associated with hypertension [28, 50]. In the present study, 4-PBA reduced proteinuria in rats with PHN

when the treatment was initiated either at the time of disease onset or during the established phase of the disease. This result implies that 4-PBA improved podocyte function and glomerular permselectivity. In parallel, 4-PBA reduced the excretion of ERdj3 and MANF in the urine.

A question that arises is whether urinary ERdj3 and MANF were secreted exclusively from podocytes in PHN. Some KDEL chaperones were increased not only in glomeruli, but also in kidney tubular fractions, suggesting induction of tubular ER stress. MANF increased only in glomeruli, but not tubules. Tubular ER stress could potentially be a result of proteinuria; however, it should be noted that comparable levels of ERdj3 and MANF were excreted in the urine in rats with PHN and PAN, even though PHN rats had ~15-fold greater urinary protein excretion. Furthermore, there was weak or at most moderate correlation between the amounts of urinary chaperone excretion and levels of proteinuria, indicating that the two are most likely unrelated. Alternatively, in PHN, circulating nephritogenic anti-Fx1A antibody could pass into the urine and bind to tubular brush border to induce stress. Mice with cystinosis, a tubulopathy associated with ER stress [38], did not show an increase in urinary ERdj3. Thus, tubular injury apparently does not lead to increased urinary ER chaperones, although some secretion of chaperones by tubular cells secondary to a primary glomerular injury cannot be excluded. In the present study, we did not reliably detect urinary ERdj3 and MANF in PHN before the onset of proteinuria. Perhaps, immunoblotting is not sufficiently sensitive to detect secretion of these ER chaperones at low levels, and more sensitive assays (e.g., ELISA) may require consideration in the future.

In GECs, ERP57 and GRP94 were not secreted. While this may be expected, there are some exceptions to this rule. A previous study showed that ERP57 could be secreted extracellularly after stimulation of cells by transforming growth factor-β (TGFβ), but not TM [51]. TGFβ is a profibrogenic factor, and in this study, the authors also showed that ERP57 can be detected in the urine of mice with renal fibrosis, as well as patients with diabetic nephropathy and microalbuminuria [51]. Possibly the interaction of ERP57 with specific secretory client proteins (i.e., extracellular matrix components) may lead to co-secretion of the chaperone.

Although our study of ERdj3 and MANF focused on evaluating their potential as ER stress biomarkers, it should be noted that physiological roles have been ascribed to these proteins. ERdj3 is transcriptionally upregulated as a part of the UPR [46, 47, 52], and secreted ERdj3 can bind misfolded proteins in the extracellular space, thereby inhibiting protein aggregation and proteotoxicity. Mutations in ERdj3 impair the maturation and trafficking of polycystin-1, and lead to an atypical form of polycystic kidney disease [53]. MANF was shown to have a paracrine neurotrophic effect

in dopaminergic neurons [12, 54], and it may have cyto-protective effects following injury in neurons and cardio-myocytes [55, 56]. Extracellular MANF could function in an autocrine and/or paracrine capacity to protect GECs from injury in response to ER calcium depletion [45, 57].

Treatment of human MN and FSGS has tended to rely on nonspecific immunosuppressive agents, which are also associated with significant toxicity. Blockade of the renin–angiotensin system is partially effective in reducing the progression of glomerular injury. However, additional therapeutic approaches to reduce injury are desirable. The recognition of protein misfolding and ER stress in experimental glomerular diseases opens the door for testing of pharmacological agents to modulate ER stress pathways or protein quality control. 4-PBA has already been employed with a good safety profile in humans [58], although such chemical chaperones tend to be drugs of low affinity. There are, however, emerging drugs that target the ER/UPR [5, 7, 11, 57] and these may represent a promising mechanism-based approach toward attenuating podocyte injury and arresting the progression of MN, FSGS, and other glomerular diseases in humans. Reliable biomarkers are necessary to establish a diagnostic and therapeutic approach. Our "proof of concept" study shows that ameli-oration of ER protein misfolding by chemical chaperones reduces proteinuria and urinary biomarkers of ER stress will facilitate development of diagnostic and personalized therapeutic approaches to glomerular disease. A limitation of the present study is that our experimental models are not optimal for testing if urinary biomarkers of ER stress predict disease progression or decline in renal function, and further studies will be required to address these questions in the future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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