Knockdown of RTN1A attenuates ER stress and kidney injury in albumin overload-induced nephropathy

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In a recent study (7), we found that increased expression of reticulin (RTN1) was associated with the progression of CKD and inversely correlated with kidney function (as assessed by the estimated glomerular filtration rate) in patients with diabetic nephropathy (DN). RTN1 belongs to a family of RTN proteins (RTN1–RTN4), which were first described in neuroendocrine cells to localize primarily to the ER membrane as ER-shaping proteins (8, 25). RTN4, also named Nogo, has previously been the only well-characterized member in the RTN family that has been shown to induce apoptosis, inhibit neuronal regeneration, and regulate protein trafficking (1, 8, 17). Our results showed that of the three RTN1 isoforms (RTN1A, RTN1B, and RTN1C), only RTN1A expression was increased in the kidney of both human and murine models of kidney disease and that the intensity of RTN1A staining in the tubulointerstitial compartment was correlated with the progression of DN. In vitro, RTN1A mediated tunicamycin- or hyperglycemia-induced ER stress and apoptosis in kidney cells. In vivo knockdown of RTN1A expression attenuated ER stress and kidney injury in mice with unilateral ureter obstruction or diabetes. These data demonstrated an important role of RTN1A in ER stress and kidney injury.

Based on these findings, the present study was designed to test whether RTN1A mediates albuminuria-induced ER stress and apoptosis of renal tubular epithelial cells leading to the progression of CKD. Our data, indeed, show a critical role of RTN1A in the progression of albumin-induced nephropathy and suggests RTN1A as a potential target to treat renal tubulointerstitial injury induced by albuminuria in patients with proteinuric kidney disease.

MATERIALS AND METHODS

Creation of tubular cell-specific RTN1A knockdown mice. We developed a doxycycline (Dox)-inducible RNA inhibition (RNAi) model for Rtn1 in a mixed background, as previously described (8). Briefly, two lines of genetically engineered Col-TGM-Rtn1RNAi mice, each corresponding to one of the two guide sequences that achieved >50% knockdown in vitro, were generated using the collagen (Col)A1 Flp/FRT recombinase-mediated targeting system. To generate renal tubular epithelial cell-specific Rtn1a knockout mice, we crossed Pax8-reverse tetracycline transactivator mice with Rtn1aRNAi mice to obtain Pax8Rtn1aRNAi mice. Mice were fed with chow containing 625 mg/kg Dox (Bio-Serv, Frenchtown, NJ) to induce RTN1A knockdown in tubular cells.

Creation of the albumin overload nephropathy model by BSA injection. Protein overload nephropathy was induced in Pax8; Rtn1aRNAi mice and mice expressing shRNA against firefly luciferase (Pax8;LucRNAi) at 9 wk of age by an intraperitoneal injection of BSA (Sigma A-7906, Sigma Chemical, St. Louis, MO) at a dose of 10 mg/g daily for 6 consecutive weeks. BSA was dissolved in saline with a

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KO590–KO157, 2016. First published January 6, 2016; doi:10.1152/ajprenal.00485.2015. —Our previous studies have suggested a critical role of reticulon (RTN)1A in mediating endoplasmic reticulum (ER) stress in kidney cells of animal models and humans with kidney diseases. A large body of evidence suggests that proteinuria itself can cause tubular cell injury leading to the progression of kidney disease. In the present study, we determined whether RTN1A mediates proteinuria-induced tubular cell injury through increased ER stress. We found that incubation of HK2 cells with human serum albumin induced the expression of RTN1A and ER stress markers, whereas knockdown of RTN1A expression attenuated human serum albumin-induced ER stress and tubular cell apoptosis in vitro. In vivo, we found that tubular cell-specific RTN1 knockdown resulted in a significant attenuation of ER stress, tubulointerstitial injury and renal fibrosis in a model of albumin overload nephropathy. Based on these findings, we conclude that RTN1A is a key mediator for proteinuria-induced tubular cell toxicity and renal fibrosis.

Endoplasm reticulum (ER) stress has been shown to contribute to tubular cell injury and interstitial fibrosis (2, 19). Studies have shown that patients with higher levels of proteinuria are at a greater risk of progression to end-stage renal disease (20, 24). However, the underlying mechanisms of albumin-induced tubulointerstitial injury are not completely understood. Currently, there is no specific and effective therapy available to reduce the progression of chronic kidney disease (CKD) induced by proteinuria.

Endoplasmic reticulum (ER) stress has been shown to contribute to the development and progression of CKD (9, 10). Cells respond to ER stress by activating the unfolded protein response (UPR), and glucose-regulated protein of 78 kDa (GRP78) serves as a central regulator of three main UPR sensors, namely, activating transcription factor (ATF)6, inositol-requiring enzyme (IRE)-1α, and protein kinase RNA-like ER kinase (PERK), that initiate the UPR signaling pathway under ER stress. Increased expression of these ER stress sensors was observed in the tubulointerstitial compartment of human kidneys with proteinuric kidney diseases such as diabetic nephropathy and human immunodeficiency virus-associated nephropathy (7, 12).

PROTEINURIA has been postulated to contribute to tubular cell injury and interstitial fibrosis (2, 19). Studies have shown that patients with higher levels of proteinuria are at a greater risk of progression to end-stage renal disease (20, 24). However, the underlying mechanisms of albumin-induced tubulointerstitial injury are not completely understood. Currently, there is no specific and effective therapy available to reduce the progression of chronic kidney disease (CKD) induced by proteinuria.

final concentration of 0.33 mg/ml. Control Pax8;Rtn1aRNAi and Pax8;LuciRNAi mice were injected with saline. All mice were euthanized at 15 wk of age. Animal experiments were performed in accordance with guidelines of and were approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai (New York, NY).

Histology and morphological analysis. Kidney samples were fixed in 10% formalin, embedded in paraffin, and sectioned to 4 μm thickness. Periodic acid-Schiff staining was used to exam kidney histology. Picrosirius red and Coll type I staining was performed to assess of kidney fibrosis. The amount of cortical Coll was determined by quantify the percentage of positive staining areas with picrosirius red.

Urine albumin and creatinine measurements. Urine protein was measured using a commercial assay ELISA kit (Bethyl Laboratory, Houston, TX). Urine creatinine levels were quantified using a Quan-Chrom Creatinine Assay Kit (DICT-500, Bioassay Systems). Urine albumin excretion was expressed as albumin-to-creatinine (alb/cr) ratio.

Measurement of caspase-3 activity by ELISA. Caspase-3 activity was measured in HK2 cells using a Human Active Caspase-3 Immunoassay Kit (R&D Systems) following the manufacturer’s protocol.

Cell culture. HK2 cells were obtained from the American Type Culture Collection and cultured as previously described (31). Cells were stimulated with endotoxin-free human serum albumin (HSA; Sigma-Aldrich).

Lentiviral preparation and infection. Different clones of lentivectors expressing short hairpin (sh)RNA for RTN1 were obtained from Open Biosystems. Human embryonic kidney (HEK)-293T cells were transfected with either lentiviral plasmid expressing the RTN1 shRNA sequence pGIPZ-shRTN1 or control scrambled sequence (pGIPZ-scramble) plus psPAX2 packaging plasmid and pMD2.G envelop

Fig. 1. Knockdown of reticulon (RTN)1A inhibited human serum albumin (HSA)-induced endoplasmic reticulum (ER) stress and apoptosis in HK2 cells. A: Western blots and real-time PCR analysis of RTN1A expression in HK2 cells transduced with lentiviral constructs expressing short hairpin (sh)RNAs against RTN1A [clone 4 (CL4) or mixtures of clones 1 and 4 (CL1+4)] or scrambled (Scr) shRNA as a control. Four days after transduction, HK2 cells were incubated with HSA (20 mg/ml) for an additional 48 h. B: expression of ER stress markers as quantified by real-time PCR. GRP78, glucose-regulated protein of 78 kDa; CHOP, C/EBP homologous protein. C: bar graph of cleaved caspase-3 concentrations for the experimental conditions described above. n = 3. *P < 0.01 compared with Scr shRNA (−HSA); #P < 0.01 compared with Scr shRNA (+ HSA).
plasmid using PolyJet transfection reagent according to the manufacturer’s protocol (SignaGen Laboratories). Forty-eight hours after transfection, lentiviral particles were harvested from te HEK-293T cell culture medium. Concentrated lentiviral particles were used to infect HK2 cells. The efficiency of knockdown was confirmed by both Western blot analysis and real-time PCR.

Quantitative real-time PCR. Primers for RT-PCR were designed using the Primer-Blast (National Center for Biotechnology Information, Bethesda, MD) tool. Gene expression was normalized to the housekeeping gene GAPDH, and fold changes in expression relative to the respective shRNAs in renal tubules. Our previous work showed a nearly 70% reduction of RTN1A mRNA and protein expression in kidney cortexes of Pax8;Rtn1aRNAi mice compared with Pax8;LuciRNAi mice during the induction of the respective shRNAs in renal tubules. Various primers were used for each experiment (n = 3–5 experiments).

Western blot analysis. Protein lysate preparation and Western blot analysis were performed as previously described (2). Briefly, tissues were lysed with buffer containing 1% Nonidet P-40 and a protease and phosphatase inhibitor cocktail. The specific antibodies described below were used for immunoblot analysis. Antibody against RTN1A was purchased from Abcam (catalog no. ab8957). Antibodies for phosphorylated (p-)PERK (Thr517), total PERK (catalog no. C33E10), C/EBP homologous protein (CHOP; catalog no. 2895), GRP78 (catalog no. 3177), and GAPDH (catalog no. 2118) were from Cell Signaling Technology. The density for each tested protein was normalized against GAPDH.

Statistical analysis. Data are expressed as means ± SE. ANOVA followed by the Bonferroni correction was used to analyze means between more than two groups. An unpaired t-test was used to analyze data when two groups were present. GraphPad Prism 5 software was used for statistical analyses. P values of <0.05 were considered statistically significant.

RESULTS

RTN1A mediates albumin-induced ER stress and apoptosis in HK2 cells. It has been previously demonstrated that exposure of renal tubular epithelial cells to a higher protein load in the glomerular filtrate induces ER stress and apoptosis of renal tubular cells (12). Albumin-induced ER stress in cultured tubular cells has also been previously reported (10, 11). To determine whether RTN1A is a key mediator of albumin-induced ER stress and apoptosis in renal tubular cells, RTN1A expression was knocked down in HK2 cells using a lentiviral vector expressing RTN1A shRNA [clone4 or a mixture of clone (1+4)], as we have previously described (7). After 3 days posttransduction, cells were exposed to HAS for an additional 48 h. HK2 cells infected with lentiviral vector expressing a scrambled shRNA sequence were used as a control. Consistent with our previous results of increased RTN1 expression upon renal cell injury, we found that treatment with HSA upregulated the expression of RTN1A and the ER stress response, as indicated by upregulation of the known ER stress markers GRP78 and CHOP (Fig. 1, A and B). Knockdown of RTN1A, however, attenuated HSA-induced expression of the ER stress response. In addition, HSA increased apoptosis of HK2 cells, as assessed by measurements of caspase-3 activity, which was also diminished by RTN1A knockdown (Fig. 1C). These data suggest that RTN1A mediates HSA-induced ER stress and apoptosis of tubular epithelial cells.

Generation of the albumin overload nephropathy model in tubular cell-specific Rtn1A knockdown mice. To determine the effects of albumin overload on tubular cell injury in vivo, we generated a tetracycline-inducible Rtn1RNAi mice model using the Col1A1 Flp/FRT recombinase-mediated targeting system (Col1A1-TRE-GFP-miR-shRNA), as previously described (7). To confirm a local and direct role of RTN1A in renal tubular cells, we crossed Pax8-reverse tetracycline transactivator transgenic mice with Rtn1RNAi mice to generate a tubular epithelial cell-specific RTN1A knockdown mouse model (Pax8;Rtn1aRNAi). Pax8;LuciRNAi mice were used as controls. Both Pax8;Rtn1aRNAi and Pax8;LuciRNAi mice were fed with chow supplemented with Dox at 625 mg/kg (Harlan Laboratories) starting at 6 wk of age to induce the expression of the respective shRNAs in renal tubules. Our previous work showed a nearly 70% reduction of RTN1A mRNA and protein expression in kidney cortexes of Pax8;Rtn1aRNAi mice compared with Pax8;LuciRNAi control mice after 3 wk of Dox administration (7). To induce albumin overload-induced nephropathy, after 3 wk of Dox feeding, Pax8;Rtn1aRNAi and Pax8;LuciRNAi mice received an intraperitoneal injection of BSA at a dose of 10 mg/g (or saline vehicle as a control) daily for 6 consecutive weeks. Body weight and urine and blood samples were collected at three distinct time points (1, 3, and 6 wk) after the BSA injection.

Pax8;Rtn1aRNAi mice did not develop any obvious abnormal renal or extrarenal phenotype compared with Pax8;LuciRNAi mice. There were no significant differences in body weight among the four groups except that Pax8;LuciRNAi + BSA mice weighed significantly less than Pax8;Rtn1aRNAi + BSA mice at 1 wk after the BSA injection (P < 0.05; Fig. 2). While the urinary albumin excretion rate in both BSA-injected groups (Pax8;Rtn1aRNAi + BSA and Pax8;LuciRNAi + BSA groups) was significantly higher than in vehicle-injected groups, it was significantly reduced in the Pax8;Rtn1aRNAi + BSA group compared with the Pax8;LuciRNAi + BSA group (Fig. 3). Thus,

Fig. 2. Body weight of mice. Age-matched Pax8;Rtn1aRNAi mice and Pax8;LuciRNAi mice were injected with BSA or vehicle as described in MATERIALS AND METHODS. Body weight was recorded for these mice at the indicated time points. n = 5. *P < 0.05 for Pax8;Rtn1aRNAi mice with or without BSA.
our data indicate that tubular cell-specific knockdown of RTN1A had a protective effect on albumin overload-induced kidney injury.

**RTN1A knockdown attenuates kidney fibrosis in albumin-overloaded mice.** All sustained kidney injury leads to the development of tubulointerstitial fibrosis. To ascertain the renoprotective effect of RTN1A knockdown in the progression of albumin-induced nephropathy, we performed histological and immunohistological examinations for renal fibrosis in kidneys obtained from all four groups using periodic acid-Schiff, picrosirius red, and Col type 1 immunostaining. We found that Pax8;Rtn1aRNAi + BSA mice indeed had a significantly attenuated extent of tubulointerstitial fibrosis, tubular dilatation, and mononuclear cell infiltrates compared with Pax8;LuciRNAi + BSA mice (Fig. 4). Consistently, kidneys of Pax8;Rtn1aRNAi + BSA mice also showed suppressed mRNA levels of renal fibrosis markers, including Col type 1, α-smooth muscle actin, fibroblast-specific protein-1, and fibronectin (Fig. 5). Taken together, these results provide further evidence that a reduction in RTN1A in tubular cells abrogates the development of tubulointerstitial fibrosis, which is a key event in the progression of CKD.

**RTN1A knockdown attenuates ER stress response in albumin-overloaded mice.** To determine whether the renoprotection by RTN1A knockdown in albumin-overloaded mice involves

![Fig. 4: RTN1A knockdown attenuated kidney injury and renal fibrosis in albumin-overloaded mice. Pax8;Rtn1aRNAi and Pax8;LuciRNAi mice were fed doxycycline (Dox) for 3 wk before BSA injection. A: mice were euthanized at 6 wk after BSA or vehicle injection, and kidneys were removed for periodic acid-Schiff (PAS), picrosirius red, and collagen type 1 staining. Representative images from five mice in each group are shown. B: quantification of the percentage of the fibrotic area with picrosirius red staining (average of 10 fields of area/mouse, n = 5 mice/group). *P < 0.001 compared with Pax8;LuciRNAi + vehicle mice; #P < 0.001 compared Pax8;Rtn1aRNAi + BSA mice.](http://ajprenal.physiology.org/)}
the ER stress pathway, we examined the expression of ER stress markers at both protein and mRNA levels. Real-time PCR and Western blot analyses of the kidney cortex confirmed a >50% reduction in Pax8;Rtn1aRNAi mice, as we had previously observed (7) (Fig. 6). Expression of RTN1A and other known ER stress markers (GRP78, CHOP, and phosphorylation of PERK) increased in the kidneys of BSA-injected mice compared with those of vehicle-injected mice. However, their expressions were markedly lower in the kidneys of Pax8;Rtn1aRNAi mice compared with those of vehicle-injected mice. Moreover, their expressions were markedly lower in the kidneys of Pax8;Rtn1aRNAi mice compared with those of vehicle-injected mice. However, their expressions were markedly lower in the kidneys of Pax8;Rtn1aRNAi mice compared with those of vehicle-injected mice. Hence, the attenuation of renal injury in RTN1A knockdown kidneys in response to albumin overload occurs through mitigation of the ER stress response.

RTN1A knockdown attenuates apoptosis of renal tubular epithelial cells in albumin-overloaded mice. Consistent with the inhibition of a sustained ER stress response, we found that knockdown of RTN1A in tubular cells also attenuated albumin-induced apoptosis in vivo, as assessed by increased cleavage of caspase-3 in the kidney cortex by immunoblot analysis (Fig. 6, B and C) and by detection of apoptotic cells on kidney sections by TUNEL (Fig. 7). Taken together, our data demonstrate that reduced expression of RTN1A leads to a significant attenuation of tubular cell ER stress, apoptosis, and renal fibrosis in a model of albumin overload nephropathy and further indicates that RTN1A is a key mediator for proteinuria-induced tubular cell toxicity and renal fibrosis.

**DISCUSSION**

Proteinuria plays an important role in the progression of tubulointerstitial injury (10, 24). It has been recognized that excessive protein in urine is not merely a marker of the kidney injury but has intrinsic cell toxicity that contributes to tubular injury and fibrosis (28). The protein droplets seen in the cytoplasm of proximal tubular cells are associated with an acceleration of tubulointerstitial damage via the proinflammatory response (13, 27) and oxidative stress (15, 18) in proximal tubules, which, in turn, leads to the excretion of chemokines and cytokines, resulting in inflammation, transformation of interstitial fibroblasts, and fibrosis (6). Therefore, tubular epithelial cells play a central role in orchestrating the progression of proteinuria-induced renal injury and fibrosis.

The ER is known to be responsible for protein folding and maintenance of homeostasis in cells and is considered a key player in the response to cellular stress and protein overload. Recent studies have suggested the imbalance between the folding of proteins and ER capacity as a cause of apoptosis (18, 22), as an excessively prolonged UPR leads cell apoptosis (3, 16). Stimulation by albumin overload may cause an accumulation of misfolded proteins in tubular cells and induce sustained ER stress. Indeed, exposure of renal proximal tubular cells to high albumin concentrations results in ER stress and apoptosis in vitro and in vivo (21). Furthermore, elevated urinary protein excretion in humans has been shown to be...
associated with tubular injury and ER stress, leading to the development of tubulointerstitial fibrosis (4, 23). In a murine model with nephrotic syndrome, ER stress markers were elevated in kidney epithelial cells (5, 26), and ER stress regulatory proteins binding immunoglobulin protein and CHOP were found to be upregulated in the kidneys of an experimental DN model in rats (14) and associated with an increase in tubular cell apoptosis. Taken together, these studies suggest that ER stress plays a major role in tubular injury induced by albumin overload, resulting from an increase in filtered proteins.

In our previous study (7), we reported that RTN1A is expressed predominantly in the tubulointerstitial compartment and that its increased expression inversely correlated with declining estimated glomerular filtration rate in patients with DN, suggesting a role of RTN1A in tubulointerstitial injury in DN patients. Since proteinuria is the key event and a strong prognostic factor for the progression of DN, we speculated that RTN1A might mediate proteinuria-induced kidney injury in DN. Our current data now show that RTN1A mediates the ER stress response in HK2 cells to a high concentration of HSA, as detected by increased expression of GRP78, CHOP, and p-PERK. It also mediates albumin overload-induced apoptosis, likely through the activation of the ER stress response. PERK is known to be an important UPR sensor in the ER and is activated through phosphorylation under ER stress, and CHOP is a proapoptotic transcriptional factor. In our previous study (7), we observed a direct interaction between RTN1A and PERK and that this interaction is required for RTN1A-mediated activation of the ER stress and apoptosis pathway. Taken together, these data suggest a pivotal role of RTN1A in UPR signaling and apoptosis pathways in renal epithelial cells.

To confirm the role of RTN1A in the progression of kidney disease in vivo, we developed a mouse model with inducible tubular cell-specific knockdown of RTN1A. We found that reduction of RTN1A expression specifically in tubular cells ameliorated albuminuria, tubular interstitial fibrosis, and mononuclear cell infiltrates in the mouse model of albumin overload nephropathy. Consistent with the in vitro findings, knockdown of RTN1A expression also reduced the expression of ER stress markers and attenuated apoptosis after the albumin overload. These findings suggest that the increased expression of RTN1A in tubular epithelial cells shifts the response to a high concentration of albumin from an adaptive UPR to a proapoptotic pathway and that RTN1A-mediated ER stress contributes to the tubulointerstitial injury and accelerates the progression of kidney disease. While our results do not preclude a potential role of RTN1A in interstitial cells in propagation of the inflammatory response and fibrosis, it does suggest that a reduction of RTN1A expression and the ER stress response specifically in the tubular epithelial compartment is sufficient to mitigate tubulointerstitial injury and fibrosis induced by albumin overload.

In conclusion, our present study demonstrates a critical role of RTN1A in mediating proteinuria-induced tubular cell injury and the progression of kidney disease through increased ER stress and apoptosis. Our data further suggest that inhibition of RTN1A expression may be protective in kidney tubular cells and that the development of RTN1A inhibitors may be a potential therapy to prevent proteinuria-induced tubulointerstitial injury in patients with proteinuric kidney disease.

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