Biochemistry and Biophysics Reports 17 (2019) 139–150

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

TRPC6 inactivation does not affect loss of renal function in nephrotoxic serum glomerulonephritis in rats, but reduces severity of glomerular lesions

Eun Young Kim[a], Parisa Yazdizadeh Shotorbani[a], Stuart E. Dryer[a,b,*]

[a] Department of Biology and Biochemistry, University of Houston, Houston, TX, USA
[b] Department of Medicine, Division of Nephrology, Baylor College of Medicine, Houston, TX, USA

ARTICLE INFO

Keywords:
Chronic kidney disease
TRPC6
Renal fibrosis
Glomerulonephritis

ABSTRACT

Canonical transient receptor potential-6 (TRPC6) channels have been implicated in a variety of chronic kidney diseases including familial and acquired forms of focal and segmental glomerulosclerosis (FSGS) and renal fibrosis following ureteral obstruction. Here we have examined the role of TRPC6 in progression of inflammation and fibrosis in the nephrotoxic serum (NTS) model of crescentic glomerulonephritis. This was assessed in rats with non-functional TRPC6 channels due to genomic disruption of an essential domain in TRPC6 channels (*Trpc6*$^{del/del}$ rats) and wild-type littermates (*Trpc6*$^{wt/wt}$ rats). Administration of NTS evoked albuminuria and proteinuria observed 4 and 28 days later that was equally severe in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. By 28 days, there were dense deposits of complement and IgG within glomeruli in both genotypes, accompanied by severe inflammation and fibrosis readily observed by standard histological methods, and also by increases in renal cortical expression of multiple markers (α-smooth muscle actin, vimentin, NLRP3, and CD68). Tubulointerstitial fibrosis appeared equally severe in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. TRPC6 inactivation did not protect against the substantial declines in renal function (increases in blood urea nitrogen, serum creatinine and kidney:body weight ratio) in NTS-treated animals, and increases in a urine maker of proximal tubule pathology (β2-macroglobulin) were actually more severe in *Trpc6*$^{del/del}$ animals. By contrast, glomerular pathology, blindly scored from histology, and from renal cortical expression of podocin suggested a partial but significant protective effect of TRPC6 inactivation within the glomerular compartment, at least during the autologous phase of the NTS model.

1. Introduction

Canonical transient receptor potential-6 (TRPC6) channels are Ca^{2+}-permeable cationic channels that play complex roles in the regulation of cellular physiology in excitable and non-excitable cells [1]. TRPC6 is expressed in several cell types in the kidney, especially in podocytes and mesangial cells, and possibly also in renal tubular cells and in renovascular smooth muscle [reviewed in 1]. TRPC6 is also expressed in a variety of immune cells, including macrophages [2], T-cells [3], and neutrophils [4]. The normal physiological function of TRPC6 in renal cells is not understood. However, it seems likely that they contribute to contractile responses in mesangial cells [5] and they appear to regulate cytoskeletal dynamics [6] and transcriptional responses [7,8] in podocytes.

There is extensive evidence that TRPC6 channels can be dysregulated in kidney diseases. It was initially discovered that numerous mutations in TRPC6 are associated with familial forms of focal and segmental glomerulosclerosis (FSGS) [9–12]. Most but not all of these mutations cause a gain of function in TRPC6 channels when they are expressed in heterologous expression systems, owing to increased or sustained activation during signal transduction cascades and/or increased steady-state abundance on the cell surface [9–12]. There is also evidence that TRPC6 is dysregulated in acquired (non-inherited) kidney diseases. For example, TRPC6 protein and transcript abundance is increased in glomeruli from patients with primary FSGS, minimal change disease, and membranous glomerulonephritis [13]. More recently, we

Abbreviations: BUN, blood urea nitrogen; CKD, chronic kidney disease; FSGS, focal and segmental glomerulosclerosis; GBM, glomerular basement membrane; IL-1β, interleukin 1β; NLRP3, NOD-like receptor pyrin domain containing-3 protein; NTS, nephrotoxic serum; PAS, periodic acid-Schiff's stain; PAN, puromycin amino nucleoside; SMA, α-smooth muscle actin; suPAR, soluble urokinase receptor; TCA, trichloroacetic acid; TNF, tumor necrosis factor; TRPC3, canonical transient receptor potential-3 channel; TRPC5, canonical transient receptor potential-5 channel; TRPC6, canonical transient receptor potential-6 channel; UUO, unilateral ureteral obstruction

* Correspondence to: Department of Biology and Biochemistry, University of Houston, Houston, TX 77204-5001, USA.
E-mail address: sdryer@uh.edu (S.E. Dryer).

https://doi.org/10.1016/j.bbrep.2018.12.006
Received 16 October 2018; Received in revised form 6 December 2018; Accepted 16 December 2018
Available online 08 January 2019

have observed that serum and plasma from patients with recurrent FSGS is able to evoke a marked increase in the abundance of TRPC6 channels on the podocyte cell-surface, accompanied by increased activation of the channels by mechanical stimuli [14]. This up-regulation of TRPC6 is mimicked by circulating factors associated with chronic kidney diseases such as the soluble urokinase receptor (suPAR) and tumor necrosis factor (TNF), which appear to act on podocytes in an additive or synergistic manner [14,15].

TRPC6 contributes to the progression of glomerular disease in animal models [16–19]. The most robust evidence for this comes from studies of chronic puromycin aminonucleoside (PAN) nephrosis in rats, a widely used model of severe secondary forms of FSGS [20]. We observed that genetic inactivation of TRPC6 in Sprague-Dawley rats, accomplished by deletion of an essential portion of the channel molecule by CRISPR/Cas9 technology (producing an allele hereafter referred to as *Trpc6*^{del}), markedly reduced albuminuria, glomerulosclerosis, and ultrastructural changes in glomeruli that occurred during the chronic phase of PAN nephrosis [19]. TRPC6 knockout also reduced tubulointerstitial fibrosis during chronic PAN nephrosis, and reduced the number of immune cells that infiltrated into glomeruli [19]. The later observations raise the possibility that at least a portion of the protective effects of TRPC6 inactivation in chronic PAN nephrosis arise from some degree of immunosuppression. TRPC6 channels are expressed and functional in active immune cells, and they may also play a role in allowing leukocytes to traverse endothelial layers of microvasculature [21,22]. They also play a role in the transdifferentiation of myofibroblasts [23], an essential process during renal fibrosis. Therefore it is interesting that TRPC6 knockout in mice is sufficient to markedly reduce interstitial fibrosis evoked by unilateral ureteral obstruction, and inhibition of TRPC6 may underlie protective effects of soluble klotho seen in this model [24]. These observations raise the possibility that TRPC6 could be a therapeutic target for a wide range of kidney disorders.

In the present study, we have examined a possible role for TRPC6 in driving an explicitly autoimmune rapidly-progressing glomerulonephritis, the passive nephrotoxic serum (NTS) model in Sprague-Dawley rats [25,26]. In this model, naïve animals are injected with NTS, an anti-rat glomerular basement membrane serum, in this case prepared in sheep. This results in deposition of immune deposits within glomeruli, which subsequently leads to rapid development of crescentic glomerulonephritis and severe tubulointerstitial disease that we quantified 28 days after administration of NTS. We have observed that the overall severity of kidney disease in *Trpc6*$^{del/del}$ rats was not different from wildtype *Trpc6*$^{wt/wt}$ littermates. Specifically, there was no difference in blood urea nitrogen (BUN), IgG or complement deposition in glomeruli, urine protein or albumin excretion, or in several markers of fibrosis or inflammation in serum or renal cortex, or in infiltration of immune cells into the renal cortex. However, glomerular pathology was less severe in *Trpc6*$^{del/del}$ animals compared to *Trpc6*$^{wt/wt}$ littermates. By contrast, markers of proximal tubule pathology suggested more severe disruption in *Trpc6*$^{del/del}$ rats.

2. Materials and methods

2.1. Animals

Sprague-Dawley *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats (5–6 weeks old) used in this study have been described in detail previously [19]. Briefly, the *Trpc6*^{del} allele has a 239-bp deletion in Exon 2 of the *Trpc6* gene, which results in disruption of the Ankyrin-Repeat Domain and non-functional TRPC6 channels. All procedures used in animal experiments were approved by the University of Houston Institutional Animal Care and Use Committee following NIH and ARRIVE guidelines.

2.2. Nephrotoxic Serum (NTS) model of glomerulonephritis

Sheep anti-rat isolated glomerular basement membrane (anti-GBM) serum was purchased from Probetex Inc. (San Antonio, TX). This preparation of anti-GBM serum is supplied in sterile 0.02 M phosphate buffered saline PBS, pH 7.3. It is optimized to produce severe nephritis in Sprague-Dawley rats without the need for prior sensitization to sheep antibodies. *Trpc6*$^{wt/wt}$ rats and *Trpc6*$^{del/del}$ littermates weighing 100–150 g were injected with 0.4–0.5 ml/100 gm body weight of anti-GBM serum or with 0.9% sterile saline into the tail vein. Each group was comprised of equal numbers of males and females. Urine was collected and used for determination of albumin (Exocell Inc.) and total protein (Chondrex Inc.) before NTS or saline injection, and again at 4 and 28 days after the injection. After the final urine collection, animals were weighed, a blood sample was taken, and animals were sacrificed by CO_2 inhalation followed by cervical dislocation. The kidneys were excised and weighed, and a portion of renal cortex of one kidney was removed and reserved for biochemical analysis. Portions of the other kidney were embedded in 10% buffered formalin or O.C.T. Compound™ (Tissue-Tek Inc.) for histological analysis. Serum creatinine (Exocell Inc.) and blood urea nitrogen (BUN) (ArborAssays), were determined using commercial assays.

2.3. Immunoblot analysis and enzyme-linked immunosorbent assays

Renal cortical tissue was homogenized in lysis buffer (M-PER™, Thermo Fisher Scientific) containing protease inhibitors (Sigma) and centrifuged at 13,000 rpm for 30 min. Immunoblot was carried out using standard methods. Monoclonal antibodies used in this study were: mouse anti-α-smooth muscle actin (α-SMA) clone 1A4, used at a dilution of 1:1000, from Sigma; mouse anti-vimentin (used at 1:1000) from Dako Inc.; mouse anti-CD68 (used at 1:1000) from Abcam Inc.; mouse anti-TRPC5 (used at 1:100) from NeuroMAB, Davis, CA; mouse anti-interleukin-1β (used at 1:1000) was from Santa Cruz Biotechnology. This antibody detects the precursor and mature forms of this cytokine. Polyclonal anti-rabbit NLRP3 (used at 1:1000) was from Abcam; anti-klotho (used at 1:1000) was from LSB Inc.; anti-podocin (used at 1:1000) was from Santa Cruz Biotechnology; anti-TRPC6 (used at 1:1000) and anti-TRPC3 (used at 1:1000) were from Alomone Laboratories. All experiments were repeated 3–6 times and quantified by densitometry using Image J™ software. To detect klotho in urine, samples were collected, and insoluble materials were removed by centrifugation at 2000 × *g* at 4 °C for 10 min. One volume of trichloroacetic acid (TCA) was added to 4 volumes of urine supernatant on ice for 30 min. Urine was centrifuged at 13,000 RPM for 30 min at 4 °C. The resulting protein pellet was washed three times with ice-cold acetone, and allowed to dry in the air. The dried pellets were then resuspended by boiling in 2x SDS-PAGE sample buffer and analyzed by immunoblot. Type I procollagen levels in serum were determined using a commercial assay (LSBio, Inc.) according to the manufacturer's instructions. Blood urea nitrogen (BUN) was measured by ELISA at the Baylor College of Medicine metabolic phenotyping core. Beta 2-Microglobulin levels in urine were assessed using an assay from ICL, Inc. in order to assess renal tubular injury.

2.4. Immunofluorescence

Renal tissue embedded in O.C.T. compound was snap-frozen in liquid nitrogen and cut into 6-μm sections. The deposits of rat complement C3 were evaluated by staining with fluorescein isothiocyanate-conjugated goat anti-C3 (MyBioSource Inc.), at a dilution of 1:200. To assess autologous antibody deposition, frozen sections were incubated with biotin-conjugated anti-rat IgG (R&D Systems), followed by fluorescein isothiocyanate-conjugated streptavidin (BD Biosciences). Synaptopodin was detected using a rabbit antibody from Santa Cruz Biotechnology (1:200). Pre-immune sera were used as negative staining

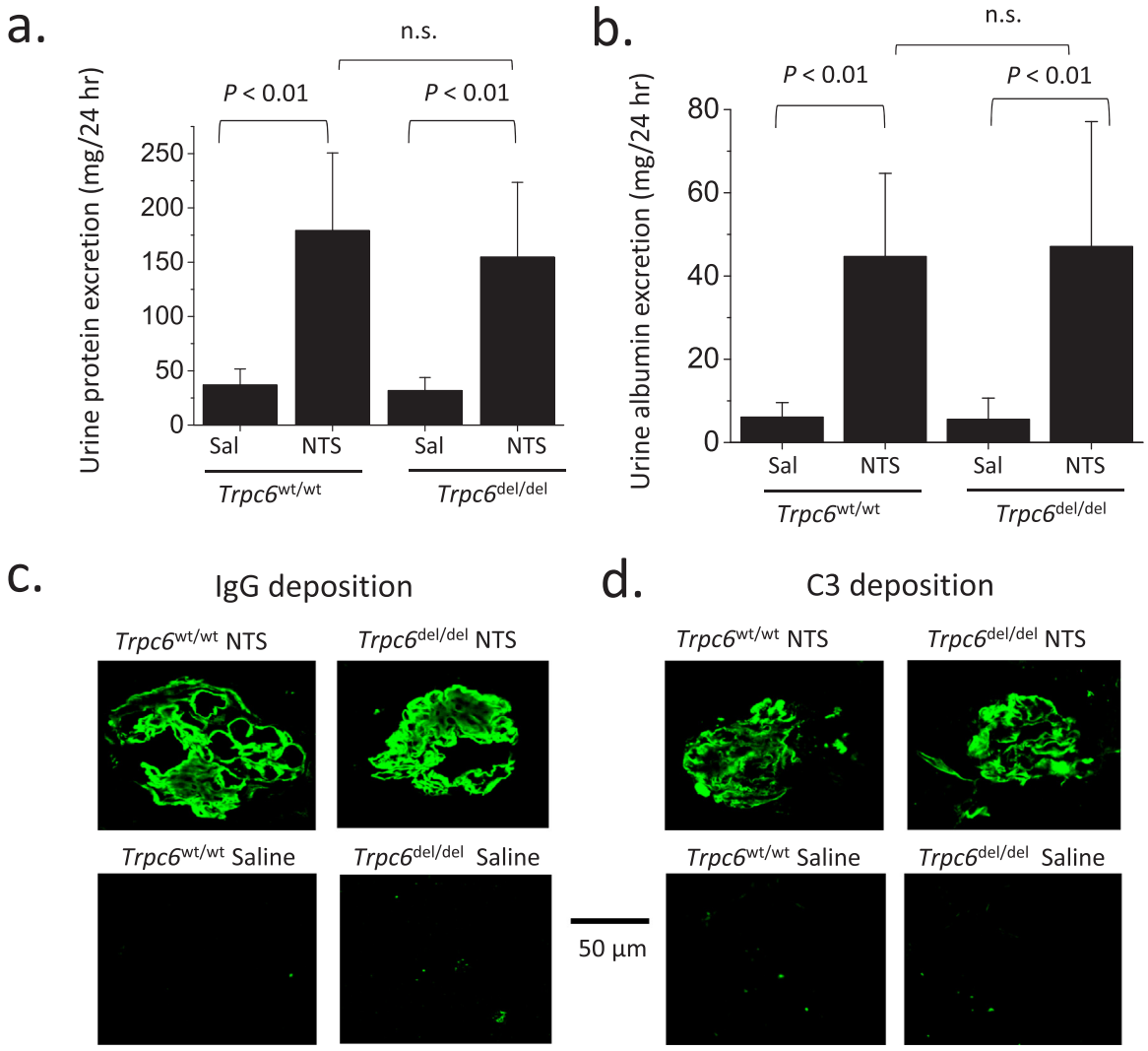

Fig. 1. Administration of NTS produces proteinuria (a) and albuminuria (b) in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. In this and all subsequent figures, bar graphs represent mean ± SD. Control rats received saline. Effects of NTS were statistically robust (N = 8 animals per group) but the genotype had no effect on responses to NTS (two-way ANOVA). NTS also resulted in deposition of IgG (c) and complement C3 (d) in glomeruli. The measurements in this figure were made 28 days after NTS or saline injection.

controls. All images were collected on an Olympus FV1000 confocal microscope.

2.5. Histopathology and immunohistochemistry

Portions of kidney from six of the animals in each group were fixed in 10% buffered formalin, embedded in paraffin, and 4-µm sections were stained with periodic acid-Schiff's (PAS) or Masson's trichrome methods as described previously [19], or by immunohistochemistry. Using PAS-stained sections, 25–50 glomeruli from each animal were blindly scored for glomerular injury as described previously [19]. Immunohistochemistry for the rat macrophage marker CD68 or for vimentin was performed on paraffin sections previously incubated with 10% H_2O_2 to block endogenous peroxidases. Sections were washed, and then treated with monoclonal anti-rat CD68 (ED-1) from Abcam (1:200 dilution) or anti-vimentin (Dako) (1:200 dilution). Immunoreactivity was visualized using the Vectastain™ ABC method (Vector Laboratories) using diaminobenzidine as a chromagen. The slides were counter-stained with hematoxylin.

2.6. Statistical analyses

All statistical analyses were carried out using public-access computational tools (http://www.vassarstats.net) with $P < 0.05$ regarded as significant. Immunoblot assays were analyzed by densitometry. The data are presented as fold changes relative to the lowest value observed in a control group, and are presented as mean ± SD. Data on 24-hr urine albumin excretion and other quantitative measures of renal function are presented as mean ± SD from $N = 8$ rats per group. However, data evaluating glomerulosclerosis in PAS stained sections were from $N = 6$ animals per group. Data were analyzed by two-way ANOVA followed by Tukey's Honest Significant Difference *post hoc* test. The two independent variables were genotype (*Trpc6*$^{wt/wt}$ versus *Trpc6*$^{del/del}$) and drug treatment (NTS versus saline vehicle). A statistically positive result was inferred when F values for the interaction between drug effects and genotype indicated $P < 0.05$.

3. Results

3.1. Analyses of renal function

The *Trpc6*$^{del/del}$ rats used in this study have been described

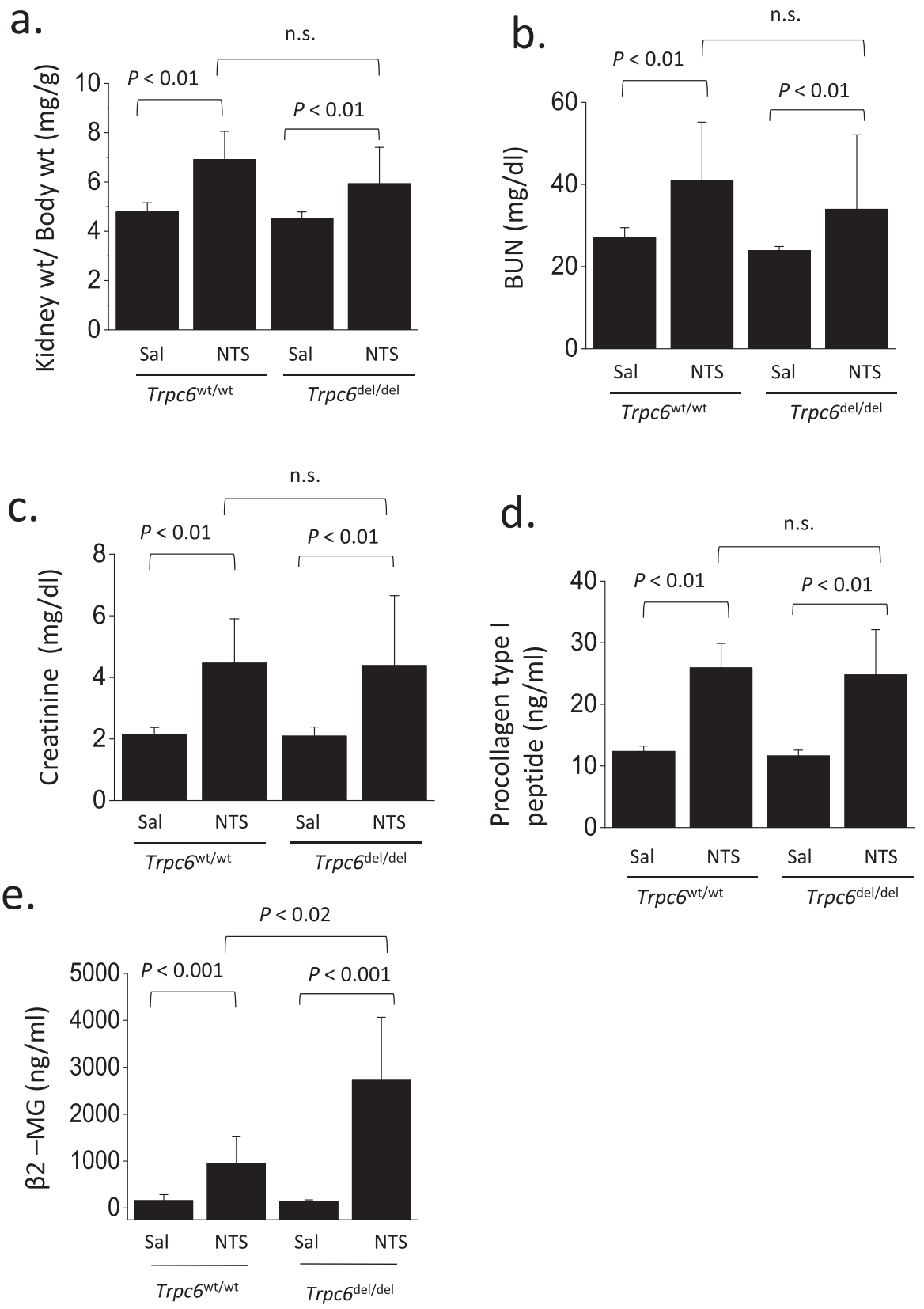

Fig. 2. TRPC6 inactivation does not protect against decline in overall renal function evoked by NTS. Administration of NTS resulted in an increase in kidney weight:body weight ratio (a), marked azotemia (b) and increases in serum creatinine (c) indicating marked decline in overall renal function. These effects were equally severe in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. In addition, serum procollagen type 1 peptide was elevated in NTS-treated animals (d), indicating comparable increases in whole-body inflammatory load in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. Urine β2-MG was increased in NTS-treated rats, and there was a significant ($P < 0.02$) effect of genotype on this response, which suggests that proximal tubule pathology is more severe in *Trpc6*$^{del/del}$ animals.

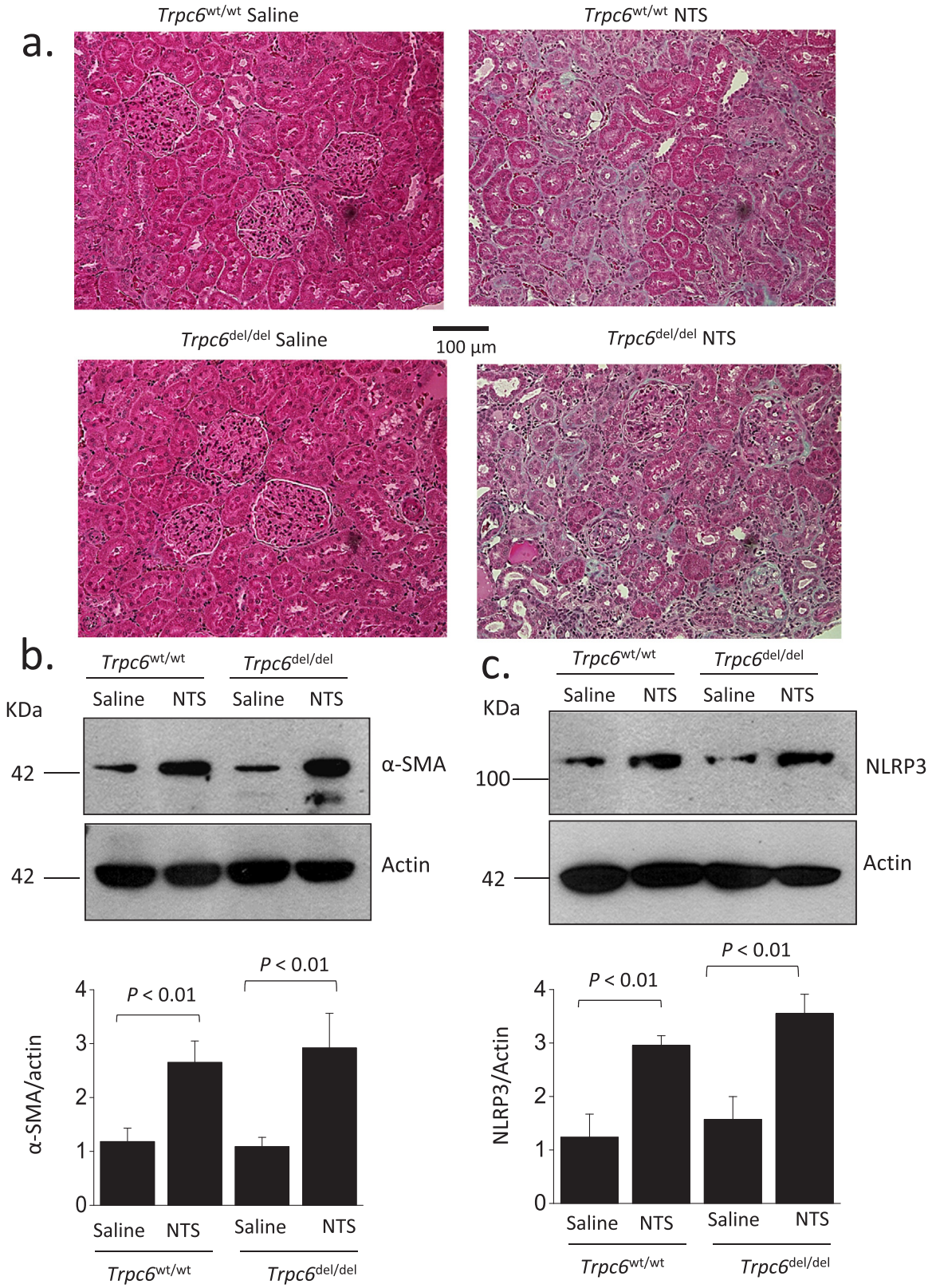

Fig. 3. NTS treatment evokes renal inflammation and fibrosis in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. This can be easily seen in sections stained by Masson's trichrome method (a). Genotype could not be discerned by this staining method in either saline- or NTS-treated animals. Increases in SMA, a marker of fibrosis (b) and NLRP3, a marker of inflammation (c) were comparable in renal cortex of NTS-treated *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. Examples of representative immunoblots are shown above densitometric analyses of these experiments.

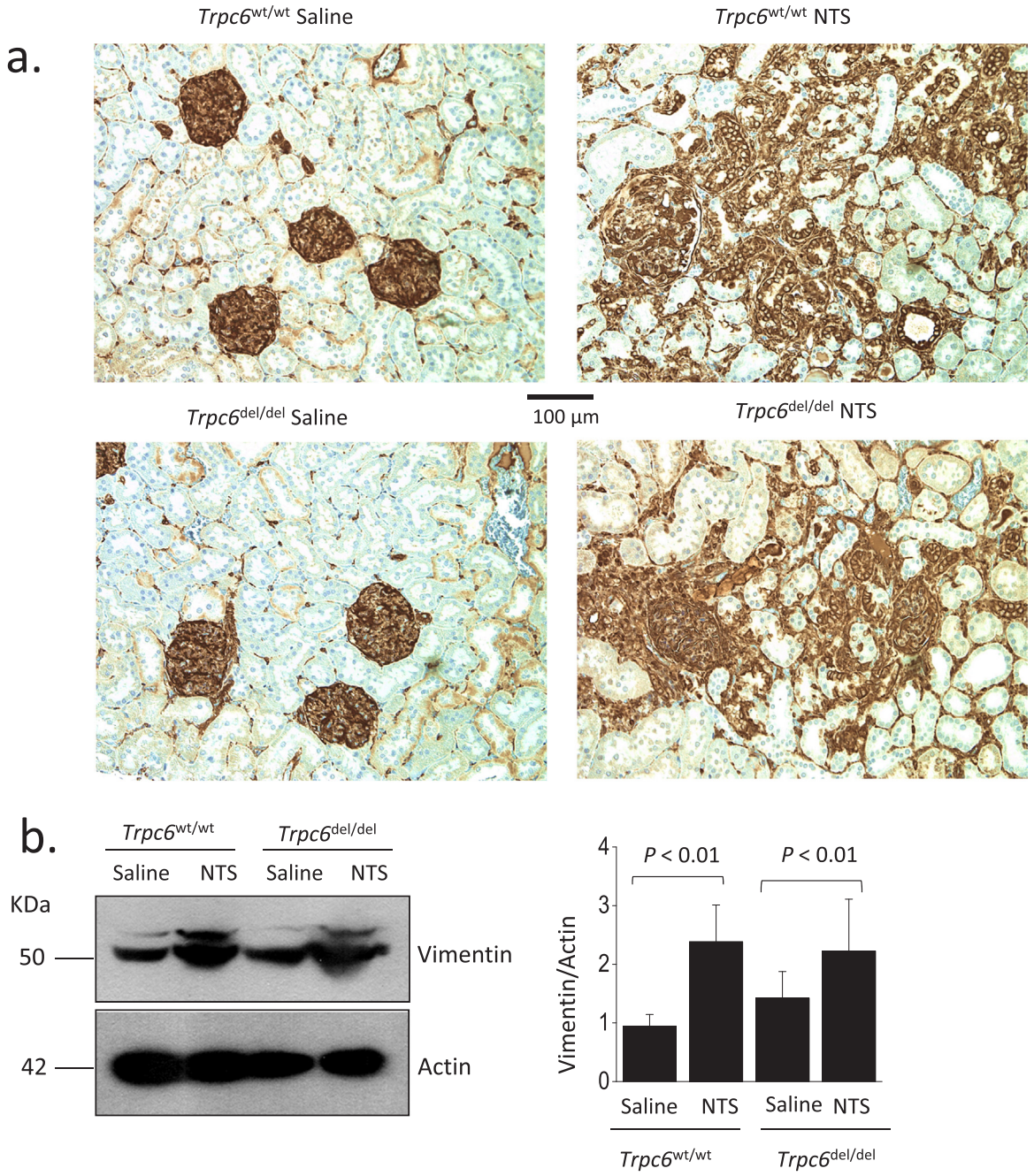

Fig. 4. Vimentin is markedly increased in tubules and interstitium of NTS-treated *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. This could be discerned by immunohistochemistry (a) and by immunoblot analysis of the renal cortex (b). Vimentin is a mesenchymal and myofibroblast maker normally expressed at high levels in glomeruli and at lower levels in the rest of the renal cortex. There was no effect of genotype on changes in this marker.

previously [19]. Briefly, the *Trpc6*^{del} allele has a 239-bp deletion in Exon 2 of the *Trpc6* gene that introduces a premature stop codon. However, the animals delete all of Exon 2 during transcription, a phenomenon that is now well documented to occur in several other genes when premature stop codons are introduced by CRISPR/Cas9 methods [27–29]. This exon skipping process allows for production of trace amounts of immunochemically detectable TRPC6 protein, but also results in disruption of the Ankyrin-Repeat Domain that is required for assembly of functional tetrameric TRPC6 channels [19,30]. In our previous study we showed that functional TRPC6 channels were not detectable in glomerular cells from *Trpc6*$^{del/del}$ rats but were readily seen in *Trpc6*$^{wt/wt}$ littermates [19]. All of our experimental designs were carried out on four groups of animals. The rats were not sensitized to sheep IgG prior to NTS injection. The treatment groups were: saline-treated *Trpc6*$^{wt/wt}$; NTS-treated *Trpc6*$^{wt/wt}$; saline-treated *Trpc6*$^{del/del}$; and NTS-treated *Trpc6*$^{del/del}$. The null-hypothesis in this experimental design was tested on the basis of a significant interaction effect between the effects of genotype and NTS treatment as determined by two-way ANOVA (or in other words, whether the effect of NTS treatment depended on the genotype). A baseline urine sample was collected when the rats were 5 weeks of age. Within the next two days rats were given a single intravenous injection of saline or NTS. The rats that received NTS had a marked increase in albumin excretion detected in urine samples collected 4 days after the injection, which is within the heterologous phase of the immune response (Supplemental data). Proteinuria and albuminuria were still severe at 28 days after the NTS injection, a time that is far into the autologous phase of the immune response (Fig. 1a, b). By that time extensive rat IgG (Fig. 1c) and complement C3 (Fig. 1d)

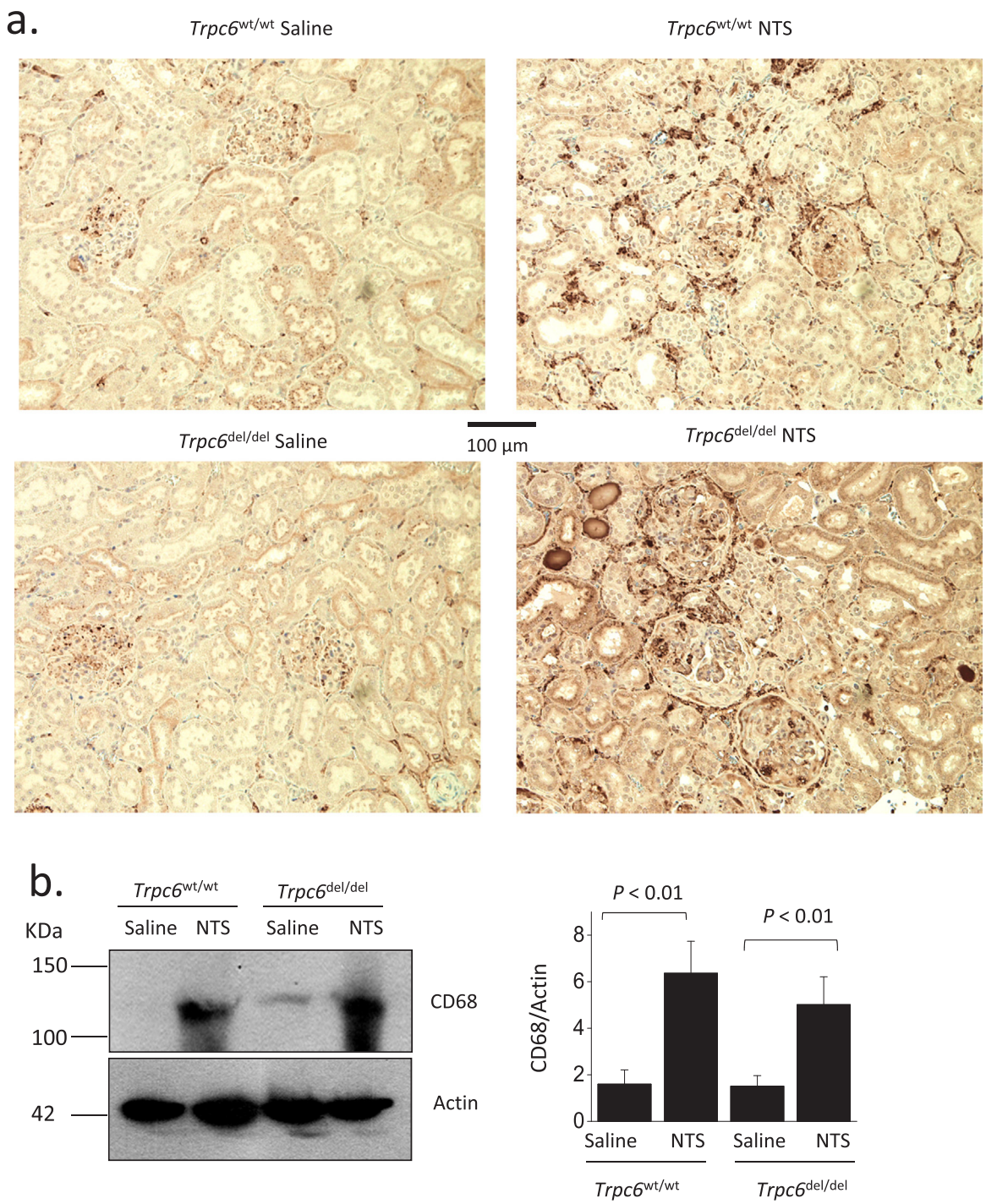

Fig. 5. Increases in macrophages or monocytic phagocytes in NTS-treated *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. This could be seen by analyzing the expression of CD68 using a monoclonal antibody known as ED-1 in immunohistochemistry (a) and by immunoblot of renal cortex (b). There was no effect of genotype on changes in this marker.

deposits were readily seen in glomeruli from NTS-treated *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats but not from saline-treated animals, and it was not possible to discern the genotype of the animals from the appearance of those deposits.

Beyond urine albumin excretion, there was clear evidence of a decline in overall renal function in NTS-treated rats of both genotypes (Fig. 2). Thus we observed increases in kidney weight:body weight ratios (Fig. 2a), BUN (Fig. 2b) and serum creatinine (Fig. 2c) in NTS-treated animals, indicating an overall decline in renal function. These deficits were equally severe in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ littermates and two-way ANOVA revealed robust effects of NTS treatment but no significant interaction effect between NTS and genotype. Serum procollagen type-1 peptide was elevated in all NTS-treated animals regardless of phenotypes, suggesting the presence of ongoing fibrotic processes, and this was not alleviated in *Trpc6*$^{del/del}$ rats (Fig. 2d). Therefore no protective effect of TRPC6 inactivation could be discerned from these markers. Indeed, urine levels of β2-microglobulin, a low-molecular weight marker for proximal tubule pathology [31], were markedly elevated in all of the NTS treated animals, but this was actually more severe in *Trpc6*$^{del/del}$ rats ($P < 0.01$ by two way ANOVA)

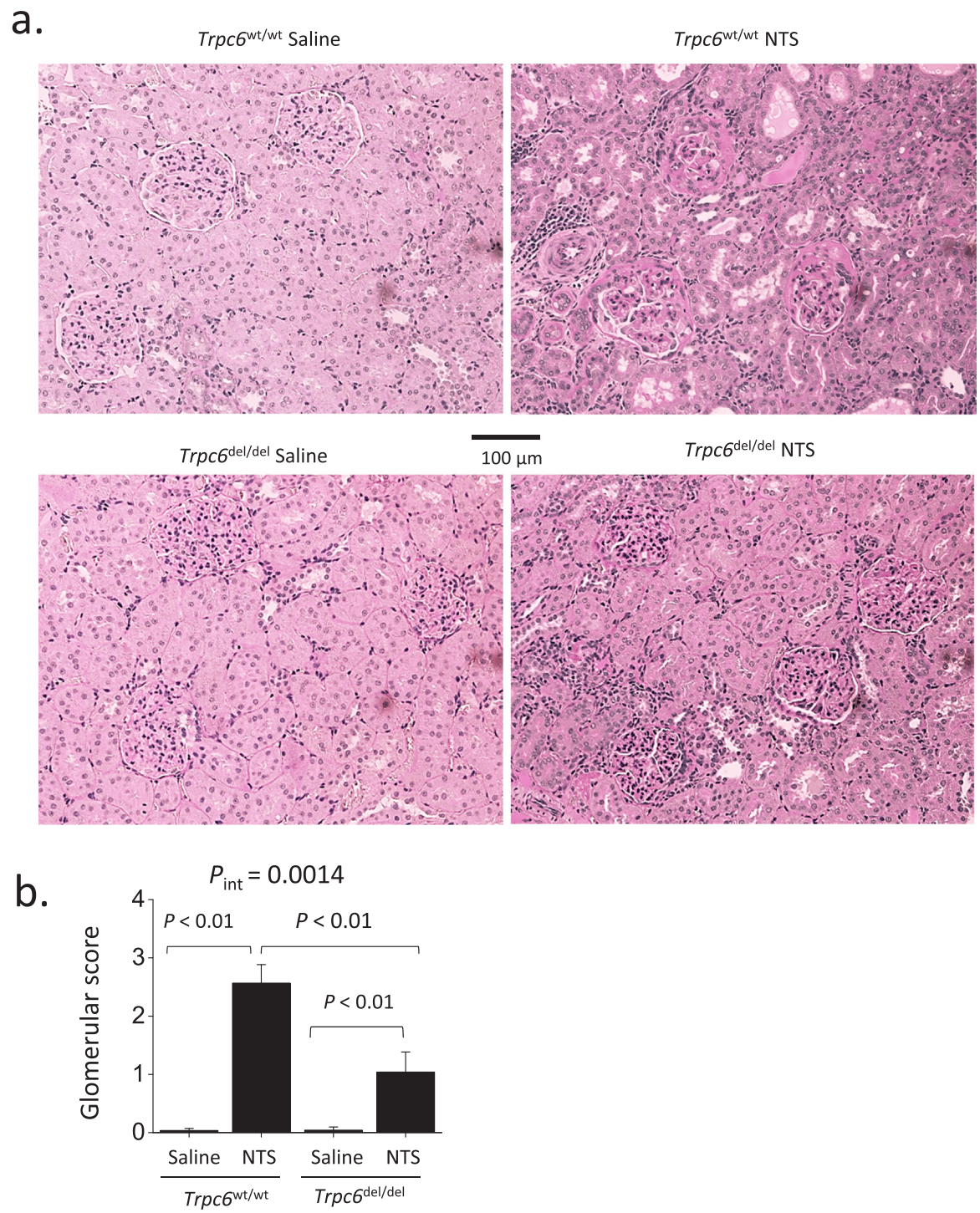

Fig. 6. Glomerulosclerosis is less severe in NTS-treated in *Trpc6*$^{del/del}$ rats compared to *Trpc6*$^{wt/wt}$ littermates. This was assessed in PAS-stained paraffin sections. Representative examples of sections are shown in (a). In NTS-treated animals note the presence of glomerulosclerosis, protein casts in tubules, hypercellularity in the interstitium, and signs of tubular necrosis. To quantify effects in glomeruli, a glomerular score (GS) on a scale of 0–4 were determined from 25 to 50 glomeruli in each animal by an observer blind to the treatment group. The mean from those measurements provided a glomerular score for that animal. The bar graph shows the mean of the glomerular scores of the animals in each treatment group. There was a significant effect of NTS treatment, as expected. However there was also a highly significant ($P = 0.0014$) effect of genotype on the response to NTS that was revealed by two-way ANOVA. This pattern indicates that glomerulosclerosis was less severe in *Trpc6*$^{del/del}$ rats compared to *Trpc6*$^{wt/wt}$ littermates.

(Fig. 2e).

3.2. Analyses of inflammation and fibrosis markers

Fibrosis in NTS-treated rats could be visualized directly using Masson's trichrome stain, which preferentially stains collagen fibers in the kidney (Fig. 3). In NTS-treated rats we observed marked increases in collagen deposition, especially in tubulointerstitial areas, but also within glomeruli (Fig. 3a). The deposits were extensive and were accompanied by clear indications of tubular atrophy and necrosis, as well as hyalinization of some tubules. Fibrosis was further evaluated by examining biochemical markers within the renal cortex using more

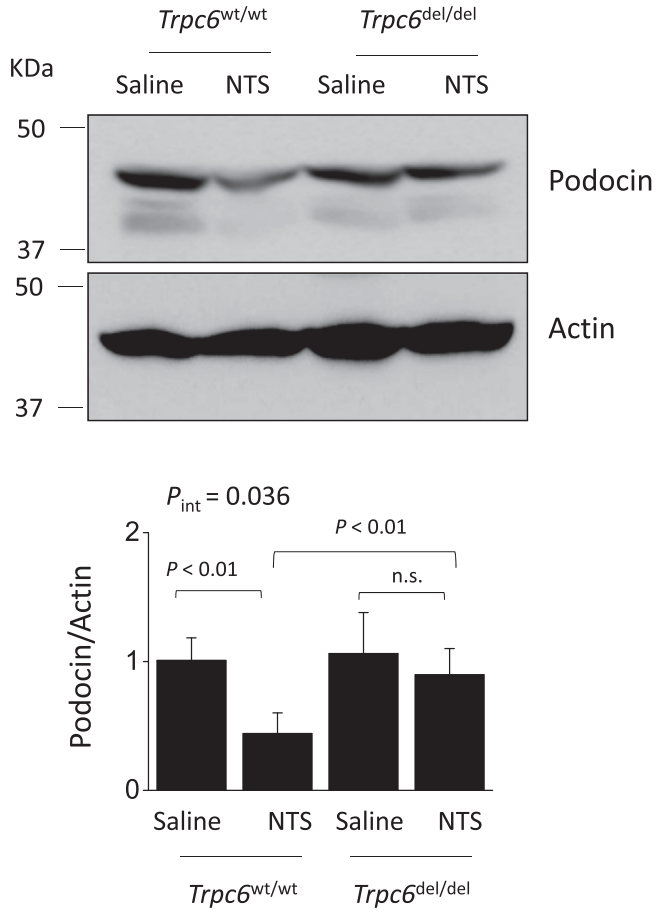

Fig. 7. Podocin abundance suggests that podocyte disease is less severe in NTS-treated in *Trpc6*$^{del/del}$ rats compared to *Trpc6*$^{wt/wt}$ littermates. This was measured by immunoblot analysis of renal cortical extracts quantified by densitometry. Protective effect of TRPC6 inactivation is revealed by significant interaction effect in two-way ANOVA ($P = 0.036$).

quantitative methods. Renal fibrosis is accompanied by increases in activated myofibroblasts and other matrix-producing cells, which can be seen as an increase in α-smooth muscle actin (SMA) abundance in renal cortex [32]. Using immunoblot analysis we observed marked increases in SMA abundance in renal cortex in NTS-treated rats, and this was equally robust in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats (Fig. 3b). We also observed marked increases in abundance of NOD-like receptor pyrin domain containing-3 protein (NLPR3) in renal cortex of NTS-treated rats (Fig. 3c). NLPR3 can drive both inflammation and fibrosis in kidney [33,34]. However there was no difference in NLRP3 abundance between *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. Consistent with this, interleukin 1β (IL-1β) was significantly increased in renal cortex of NTS-treated rats, again with no difference between *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats (Supplemental data). Active IL-1β is produced by proteolytic cleavage of a precursor secondary to activation of inflammasomes containing NLRP3 [35]. Renal fibrosis is also associated with up-regulation of mesenchymal markers such as vimentin [36]. Vimentin staining is normally confined primarily within glomeruli, as we observed in saline-treated animals by immunohistochemistry. However we detected many vimentin-expressing cells within the renal cortex in NTS-treated rats, consistent with ongoing fibrosis, and this was seen in both *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats (Fig. 4a). Vimentin was also markedly increased when measured by immunoblot from renal cortical extracts and there was no difference between *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats (Fig. 4b). Glomerulonephritis is typically characterized by activation and infiltration of macrophages and monocytes, which can be detected in rats using a monoclonal antibody (ED-1) against CD68, a marker for macrophages. We observed marked increases in abundance of CD68 in NTS-treated rats by both immunohistochemistry (Fig. 5a) and by immunoblot analysis using ED-1 (Fig. 5b). The increase in CD68 abundance was indistinguishable in *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats. These data are consistent with severe renal fibrosis in kidneys of rats that received NTS, and we did not discern any protective effect of TRPC6 inactivation.

We also examined paraffin sections obtained from six animals in each of these treatment groups using PAS staining. Consistent with observations already noted, there were indications of severe tubulointerstitial disease in all of the NTS-treated animals. These included regions of interstitial hypercellularity as well tubular atrophy, hyalinization and necrosis (Fig. 6a). Crescentic lesions in glomeruli were frequently seen. In the *Trpc6*$^{wt/wt}$ rats that received NTS, virtually every glomerulus was affected to some extent, with sclerotic lesions and/or crescents. Given our previous data showing comparable declines in renal function in both genotypes, we were somewhat surprised that glomerular lesions, while certainly present in *Trpc6*$^{del/del}$ rats that received NTS, were on average less severe, and it was possible to find glomeruli from NTS-treated *Trpc6*$^{del/del}$ rats that were unaffected or only slightly affected. Results of a semi-quantitative analysis of glomeruli carried out by a blind observer are shown in Fig. 6b. Examples of representative glomeruli illustrating the scoring system are shown in Supplemental data. This analysis revealed a statistically significant interaction between the effects of NTS treatment and genotype ($P < 0.01$), indicating a protective effect of TRPC6 inactivation within the glomerular compartment. Examples of glomeruli stained for synaptopodin from frozen sections prepared from animals in the various treatment groups are shown in Supplemental data. Consistent with the histological pattern, we also observed that NTS treatment resulted in a decrease in podocin abundance in renal cortex. However the effects of NTS on podocin abundance were less severe in *Trpc6*$^{del/del}$ rats than in their wild-type littermates (Fig. 7). There was a statistically significant interaction between the effects of NTS treatment and genotype ($P < 0.01$), indicating a quite strong protective effect of TRPC6 inactivation within the glomerular compartment, since podocytes are the only glomerular cells that express podocin, a protein that is rapidly degraded in podocyte disease models [14,15,19].

3.3. Changes in TRPC subunit abundance

In our previous study of chronic PAN nephrosis, we observed up-regulation of TRPC3 and TRPC6 as a result of PAN treatment [19]. We also observed that TRPC6 inactivation by itself caused an increase in the total abundance of TRPC3 in renal cortex [19]. A similar pattern was seen in the present experiments (Fig. 8). Specifically, we observed that NTS increased the overall abundance of both TRPC6 and TRPC3 in wildtype rats, but had no effect on the abundance of TRPC5. As in our previous study [19], TRPC3 was present at higher abundance relative to actin in *Trpc6*$^{del/del}$ rats, which could reflect a compensation for TRPC6 inactivation. However, NTS treatment did not stimulate additional increases in TRPC3 abundance in *Trpc6*$^{del/del}$ rats (Fig. 8).

Finally we note that recent studies have shown a protective effect of klotho in rodent models of tubulointerstitial disease [24], and moreover that klotho acts in part by down regulating the expression of TRPC6 [17,24]. Therefore we used immunoblot to measure klotho abundance in urine and in renal cortex from the animals in this study. We did not detect klotho in the urine of any of the NTS-treated animals examined regardless of genotype (see Supplemental data) whereas robust signal was detected in urine from all saline-treated animals regardless of genotype. Klotho was also substantially more abundant in renal cortex of saline-treated treated and this pattern was not affected by genotype (see Supplemental data).

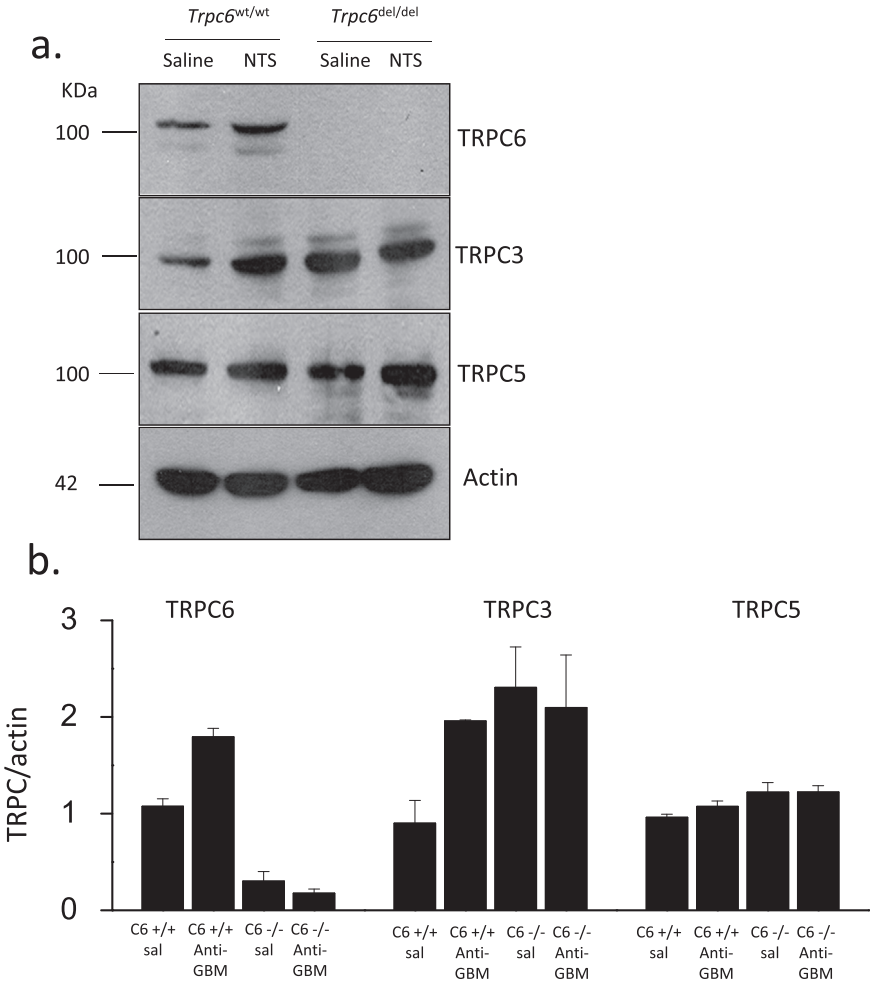

Fig. 8. Effects of NTS treatment on abundance of TRPC3, TRPC6 and TRPC5 in renal cortex. This was quantified by immunoblot from renal cortex. As described previously (Kim et al. [15,19]), TRPC3 is increased in renal cortex of *Trpc6*$^{del/del}$ rats but this does not increase further following NTS treatment. NTS increases TRPC3 and TRPC6 in *Trpc6*$^{wt/wt}$ rats. There were not changes in TRPC5 associated with genotype or NTS treatment.

4. Discussion

There is a growing body of evidence that TRPC6 channels play a role in progressive glomerular diseases, especially primary and secondary forms of FSGS [9–16,19]. In our recent study on chronic PAN nephrosis, we observed a marked attenuation of tubulointerstitial fibrosis in *Trpc6*$^{del/del}$ rats, accompanied by a reduction in the number of macrophages that infiltrated glomeruli [19]. Those effects seemed even more robust than the observed improvements in urine albumin excretion or azotemia. TRPC6 channels are known to play a role in driving activation of various immune cells, and contribute to generation of Ca^{2+} responses in macrophages [2], T-cells [3], and neutrophils [4]. They also play a role in allowing trans-epithelial migration of leukocytes [21,22]. TRPC6 channels also contribute to generation of activated myofibroblasts [23], which contribute substantially to the tubulointerstitial fibrosis that occurs following unilateral urethral obstruction in mice [36]. These observations raise the possibility that many of the beneficial effects of global TRPC6 inactivation in various rodent kidney disease models reflect suppression of immune responses and/or fibrosis pathways. If so, TRPC6 channels could represent therapeutic targets for a wide range of kidney diseases.

In the present study we have examined the effect of TRPC6 inactivation in a rodent disease model in which there is systemic immune disease triggered by formation of immune deposits on the GBM, which subsequently results in severe renal fibrosis accompanied by crescentic glomerular disease [37]. In this model we observed no protective effect of TRPC6 inactivation on overall renal function or on tubulointerstitial disease based on histology and on measurement of a wide range of markers for renal function, renal fibrosis, or local and systemic inflammation. However there appeared to be a significant protective effect of TRPC6 inactivation on the severity of glomerular damage as assessed by blinded analysis of glomerulosclerosis in histological sections and by analysis of podocin abundance. By contrast, proximal tubule pathology actually appeared worse in *Trpc6*$^{del/del}$ rats compared to *Trpc6*$^{wt/wt}$ littermates, at least based on the presence of urine markers. From this we conclude that TRPC6 inactivation is not sufficient to ameliorate autoimmune renal disease in rats.

With respect to glomerular protection, CKD inevitably places a strain on the remaining functional glomeruli, which are subjected to increased capillary pressures and single nephron glomerular filtration rates in order to sustain sufficient renal function to maintain life [38]. It is important to note that TRPC6 channels in podocytes are activated by mechanical stimuli [14,15,39] as is seen in other cell types [40–44]. If mechanical stimuli, including increased shear forces and expansion of sub-podocyte spaces, are sustained and unremitting over time, especially if accompanied by a reduction in podocin [39], the resulting chronic activation of TRPC6 would eventually lead to calcium overload in podocytes similar to that which occurs with gain-of-function mutations in TRPC6 channels in familial FSGS [9]. Calcium overload has been proposed to lead to loss or detachment of podocytes at least in part through pathways in foot processes and in the cell body that occur as a result of calcineurin and mTORC2 signaling [45]. Regardless of the

pathways involved, the protective effect of TRPC6 inactivation within the glomerular compartment does not prevent the substantial decline of renal function or the expansion of the pathology to the rest of the renal cortex in the NTS model.

Given that the initial immune deposits are formed within glomeruli, and given that TRPC6 inactivation appears to protect glomeruli, why does CKD progress to a comparable extent in *Trpc6*$^{del/del}$ rats, in marked contrast to the chronic PAN model? These disease models differ in several significant ways. In the NTS model, leukocytes activated by immune deposits within glomeruli do not remain within glomeruli [46], rather they fairly rapidly move to other compartments where they can contribute to pathological processes, especially within proximal tubules and in the interstitium. In other words, pathologically relevant processes occur in areas removed from the location of the actual immune deposits. By contrast, chronic PAN nephrosis is not mediated by adaptive immune responses and appears to occur as a result of a chain of consequences that follow an initial loss of podocytes [20]. A certain degree of glomerulosclerosis therefore occurs whenever there is a loss of functional nephrons as a result of hyperfiltration within the nephrons that remain [38,47]. This can explain why there is a reduction in glomerulosclerosis in NTS-treated *Trpc6*$^{del/del}$ rats, even though tubulointerstitial disease proceeds, and may even be more severe in proximal tubules. In this regard, we detected comparable albuminuria four days after NTS injection in both *Trpc6*$^{wt/wt}$ and *Trpc6*$^{del/del}$ rats, a time when one would not expect to observe glomerulosclerosis (Supplemental data).

TRPC6 knockout has been shown to have some protective effects in mouse models in which tubulointerstitial disease follows from an initial insult to tubules as a result of unilateral ureteral obstruction (UUO) [24]. As with chronic PAN nephrosis, the UUO model does not require activation of adaptive immune systems for fibrosis to occur [48]. It is possible that this makes UUO and chronic PAN nephrosis more responsive to TRPC6 inactivation than the NTS model. TRPC6 channels are present in collecting duct [49,50], and could play a role in the secretion of cytokines from tubules, which are especially sensitive to damage from UUO [51]. On the other hand, we cannot exclude that there is a species difference between mouse and rat, for example if TRPC3 effectively compensates for loss of TRPC6 in various immune cells, fibroblasts or myofibroblasts in rat but not in mouse.

Finally we note again that klotho was markedly decreased in renal cortex and in urine following NTS treatment. TRPC6 inactivation had no effect on this. Indeed, previous studies strongly suggest that klotho lies upstream of TRPC6 expression, and it has been suggested that the protective effect of klotho in a different disease model is due in part to reductions of TRPC6 [24]. It is certainly possible that NTS-evoked upregulation of TRPC6 seen here in wild-type rats also reflects the reduction in klotho.

5. Conclusions

We have shown that TRPC6 inactivation in rats results in protection of glomeruli in the NTS model of severe glomerulonephritis. However this does not have any effect on the decline of overall renal function or on the development of severe tubulointerstitial fibrosis in this model. TRPC6 remains a potential therapeutic target for glomerular diseases. However the role of TRPC6 in renal fibrosis appears to be model-dependent and possibly species-dependent.

Acknowledgements

This work was supported by National Institutes of Health grant R01-DK104708.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.12.006.

References

[1] A. Bouron, S. Chauvet, S.E. Dryer, J.A. Rosado, Second messenger-operated calcium entry through TRPC6, Adv. Exp. Med. Biol. 898 (2016) 201–249.
[2] V. Riazanski, A.G. Gabdoulkhakova, L.S. Boynton, R.R. Eguchi, L.V. Deriy, D.K. Hogarth, N. Loaëc, N. Oumata, H. Galons, M.E. Brown, P. Shevchenko, A.J. Gallan, S.G. Yoo, A.P. Naren, M.L. Villereal, D.W. Beacham, V.P. Bindokas, L. Birnbaumer, L. Meijer, D.J. Nelson, TRPC6 channel translocation into phagosomal membrane augments phagosomal function, Proc. Natl. Acad. Sci. USA 112 (47) (2015) E6486–E6495.
[3] C. Carrillo, A. Hichami, P. Andreoletti, M. Cherkaoui-Malki, M. del Mar Cavia, S. Abdoul-Azize, S.R. Alonso-Torre, N.A. Khan, Diacylglycerol-containing oleic acid induces increases in $[Ca^{2+}]_i$ via TRPC3/6 channels in human T-cells, Biochim. Biophys. Acta 1821 (4) (2012) 618–626.
[4] O. Lindemann, D. Umlauf, S. Frank, S. Schimmelpfennig, J. Bertrand, T. Pap, P.J. Hanley, A. Fabian, A. Dietrich, A. Schwab, TRPC6 regulates CXCR2-mediated chemotaxis of murine neutrophils, J. Immunol. 190 (11) (2013) 5496–5505.
[5] S. Sours, J. Du, S. Chu, M. Ding, X.J. Zhou, R. Ma, Expression of canonical transient receptor potential (TRPC) proteins in human glomerular mesangial cells, Am. J. Physiol. Ren. Physiol. 290 (6) (2006) F1507–F1515.
[6] K.A.T. Verheijden, R. Sonneveld, M. Bakker-van Bebber, J.F.M. Wetzels, J. van der Vlag, T. Nijenhuis, The calcium-dependent protease calpain-1 Links TRPC6 activity to podocyte injury, J. Am. Soc. Nephrol. 29 (8) (2018) 2099–2109.
[7] J. Schlöndorff, D. Del Camino, R. Carrasquillo, V. Lacey, M.R. Pollak, TRPC6 mutations associated with focal segmental glomerulosclerosis cause constitutive activation of NFAT-dependent transcription, Am. J. Physiol. Cell Physiol. 296 (3) (2009) C558–C569.
[8] T. Nijenhuis, A.J. Sloan, J.G. Hoenderop, J. Flesche, H. van Goor, A.D. Kistler, M. Bakker, R.J. Bindels, R.A. de Boer, C.C. Möller, I. Hamming, G. Navis, J.F. Wetzels, J.H. Berden, J. Reiser, C. Faul, J. van der Vlag, Angiotensin II contributes to podocyte injury by increasing TRPC6 expression via an NFAT-mediated positive feedback signaling pathway, Am. J. Pathol. 179 (4) (2011) 1719–1732.
[9] M.P. Winn, P.J. Conlon, K.L. Lynn, M.K. Farrington, T. Creazzo, A.F. Hawkins, N. Daskalakis, S.Y. Kwan, S. Ebersviller, J.L. Burchette, M.A. Pericak-Vance, D.N. Howell, J.M. Vance, P.B. Rosenberg, A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis, Science 308 (5729) (2005) 1801–1804.
[10] J. Reiser, K.R. Polu, C.C. Möller, P. Kenlan, M.M. Altintas, C. Wei, C. Faul, S. Herbert, I. Villegas, C. Avila-Casado, M. McGee, H. Sugimoto, D. Brown, R. Kalluri, P. Mundel, P.L. Smith, D.E. Clapham, M.R. Pollak, TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function, Nat. Genet. 37 (7) (2005) 739–744.
[11] S.F. Heeringa, C.C. Möller, J. Du, L. Yue, B. Hinkes, G. Chernin, C.N. Vlangos, P.F. Hoyer, J. Reiser, F. Hildebrandt, A novel TRPC6 mutation that causes childhood FSGS, PLoS One 4 (11) (2009) e7771.
[12] M. Riehle, A.K. Büscher, B.O. Gohlke, M. Kaßmann, M. Kolatsi-Joannou, J.H. Bräsen, M. Nagel, J.U. Becker, P. Winyard, P.F. Hoyer, R. Preissner, D. Krautwurst, M. Gollasch, S. Weber, C. Harteneck, TRPC6 G757D loss-of-function mutation associates with FSGS, J. Am. Soc. Nephrol. 27 (9) (2016) 2771–2783.
[13] C.C. Möller, C. Wei, M.M. Altintas, J. Li, A. Greka, T. Ohse, J.W. Pippin, M.P. Rastaldi, S. Wawersik, S. Schiavi, A. Henger, M. Kretzler, S.J. Shankland, J. Reiser, Induction of TRPC6 channel in acquired forms of proteinuric kidney disease, J. Am. Soc. Nephrol. 18 (1) (2007) 29–36.
[14] E.Y. Kim, H. Roshanravan, S.E. Dryer, Changes in podocyte TRPC channels evoked by plasma and sera from patients with recurrent FSGS and by putative glomerular permeability factors, Biochim. Biophys. Acta 1863 (9) (2017) 2342–2354.
[15] E.Y. Kim, N. Hassanzadeh Khayyat, S.E. Dryer, Mechanisms underlying modulation of podocyte TRPC6 channels by suPAR: role of NADPH oxidases and Src family tyrosine kinases, Biochem. Biophys. Acta Mol Basis Dis. 1864 (10) (2018) 3527–3536.
[16] P. Krall, C.P. Canales, P. Kairath, P. Carmona-Mora, J. Molina, J.D. Carpio, P. Ruiz, S.A. Mezzano, J. Li, C. Wei, J. Reiser, J.I. Young, K. Walz, Podocyte-specific overexpression of wild type or mutant *trpc6* in mice is sufficient to cause glomerular disease, PLoS One 5 (9) (2010) e12859.
[17] J.H. Kim, J. Xie, K.H. Hwang, Y.L. Wu, N. Oliver, M. Eom, K.S. Park, N. Barrezueta, I.D. Kong, R.P. Fracasso, C.L. Huang, S.K. Cha, Klotho may ameliorate proteinuria by targeting TRPC6 channels in podocytes, J. Am. Soc. Nephrol. 28 (1) (2017) 140–151.
[18] L. Wang, G. Jirka, P.B. Rosenberg, A.F. Buckley, J.A. Gomez, T.A. Fields, M.P. Winn, R.F. Spurney, G_q signaling causes glomerular injury by activating TRPC6, J. Clin. Investig. 125 (5) (2015) 1913–1926.
[19] E.Y. Kim, P. Yazdizadeh Shotorbani, S.E. Dryer, *Trpc6* inactivation confers protection in a model of severe nephrosis in rats, J. Mol. Med. 96 (7) (2018) 631–644.
[20] Y.H. Kim, M. Goyal, D. Kurnit, B. Wharram, J. Wiggins, L. Holzman, D. Kershaw, R. Wiggins, Podocyte depletion and glomerulosclerosis have a direct relationship in the PAN-treated rat, Kidney Int. 60 (3) (2001) 957–968.
[21] M. Tauseef, N. Knezevic, K.R. Chava, M. Smith, S. Sukriti, N. Gianaris, A.G. Obukhov, S.M. Vogel, D.E. Schraufnagel, A. Dietrich, L. Birnbaumer, A.B. Malik, D. Mehta, TLR4 activation of TRPC6-dependent calcium signaling mediates endotoxin-induced lung vascular permeability and inflammation, J. Exp. Med. 209 (11) (2012) 1953–1968.
[22] E.W. Weber, F. Han, M. Tauseef, L. Birnbaumer, D. Mehta, W.A. Muller, TRPC6 is the endothelial calcium channel that regulates leukocyte transendothelial migration

during the inflammatory response, J. Exp. Med. 212 (11) (2015) 1883–1899.
[23] J. Davis, A.R. Burr, G.F. Davis, L. Birnbaumer, J.D. Molkentin, A TRPC6-dependent pathway for myofibroblast transdifferentiation and wound healing in vivo, Dev. Cell. 23 (4) (2012) 705–715.
[24] Y.L. Wu, J. Xie, S.W. An, N. Oliver, N.X. Barrezueta, M.H. Lin, L. Birnbaumer, C.L. Huang, Inhibition of TRPC6 channels ameliorates renal fibrosis and contributes to renal protection by soluble klotho, Kidney Int. 91 (4) (2017) 830–841.
[25] D.J. Salant, A.V. Cybulsky, Experimental glomerulonephritis, Methods Enzymol. 162 (1988) 421–461.
[26] H.Y. Lan, D.J. Paterson, R.C. Atkins, Initiation and evolution of interstitial leukocytic infiltration in experimental glomerulonephritis, Kidney Int. 40 (3) (1991) 425–433.
[27] H. Mou, J.L. Smith, L. Peng, H. Yin, J. Moore, X.O. Zhang, C.Q. Song, A. Sheel, Q. Wu, D.M. Ozata, Y. Li, D.G. Anderson, C.P. Emerson, E.J. Sontheimer, M.J. Moore, Z. Weng, W. Xue, CRISPR/Cas9-mediated genome editing induces exon skipping by alternative splicing or exon deletion, Genome Biol. 18 (1) (2017) 108.
[28] J.J. Sharpe, T.A. Cooper, Unexpected consequences: exon skipping caused by CRISPR-generated mutations, Genome Biol. 18 (1) (2017) 109.
[29] T. Sui, Y. Song, Z. Liu, M. Chen, J. Deng, Y. Xu, L. Lai, Z. Li, CRISPR-induced exon skipping is dependent on premature termination codon mutations, Genome Biol. 19 (1) (2018) 164.
[30] P.K. Lepage, M.P. Lussier, H. Barajas-Martinez, S.M. Bousquet, A.P. Blanchard, N. Francoeur, R. Dumaine, G. Boulay, Identification of two domains involved in the assembly of transient receptor potential canonical channels, J. Biol. Chem. 281 (41) (2006) 30356–30364.
[31] G. D'Amico, C. Bazzi, Urinary protein and enzyme excretion as markers of tubular damage, Curr. Opin. Nephrol. Hypertens. 12 (6) (2003) 639–643.
[32] F. Strutz, M. Zeisberg, Renal fibroblasts and myofibroblasts in chronic kidney disease, J. Am. Soc. Nephrol. 17 (11) (2006) 2992–2998.
[33] W. Wang, X. Wang, J. Chun, A. Vilaysane, S. Clark, G. French, N.A. Bracey, K. Trpkov, S. Bonni, H.J. Duff, P.L. Beck, D.A. Muruve, Inflammasome-independent NLRP3 augments TGF-β signaling in kidney epithelium, J. Immunol. 190 (3) (2013) 1239–1249.
[34] G. Lorenz, M.N. Darisipudi, H.J. Anders, Canonical and non-canonical effects of the NLRP3 inflammasome in kidney inflammation and fibrosis, Nephrol. Dial. Transplant. 29 (1) (2014) 41–48.
[35] L.P. Zambetti, F. Laudisi, G. Licandro, P. Ricciardi-Castagnoli, A. Mortellaro, The rhapsody of NLRPs: master players of inflammation...and a lot more, Immunol. Res. 53 (1–3) (2012) 78–90.
[36] M.T. Grande, J.M. López-Novoa, Fibroblast activation and myofibroblast generation in obstructive nephropathy, Nat. Rev. Nephrol. 5 (6) (2009) 319–328.
[37] A.A. Eddy, Tubulointerstitial nephritis during the heterologous phase of nephrotoxic serum nephritis, Nephron 59 (2) (1991) 304–313.
[38] T.H. Hostetter, J.L. Olson, H.G. Rennke, M.A. Venkatachalam, B.M. Brenner, Hyperfiltration in remnant nephrons: a potentially adverse response to renal ablation, Am. J. Physiol. 241 (1) (1981) F85–F93.
[39] M. Anderson, E.Y. Kim, H. Hagmann, T. Benzing, S.E. Dryer, Opposing effects of podocin on the gating of podocyte TRPC6 channels evoked by membrane stretch or diacylglycerol, Am. J. Physiol. Cell Physiol. 305 (3) (2013) C276–C289.
[40] D.G. Welsh, A.D. Morielli, M.T. Nelson, J.E. Brayden, Transient receptor potential channels regulate myogenic tone of resistance arteries, Circ. Res. 90 (3) (2002) 248–250.
[41] R. Inoue, L.J. Jensen, Z. Jian, J. Shi, L. Hai, A.I. Lurie, F.H. Henriksen, M. Salomonsson, H. Morita, Y. Kawarabayashi, M. Mori, Y. Mori, Y. Ito, Synergistic activation of vascular TRPC6 channel by receptor and mechanical stimulation via phospholipase C/diacylglycerol and phospholipase A2/omega-hydroxylase/20-HETE pathways, Circ. Res. 104 (12) (2009) 1399–1409.
[42] V. Dyachenko, B. Husse, U. Rueckschloss, G. Isenberg, Mechanical deformation of ventricular myocytes modulates both TRPC6 and Kir2.3 channels, Cell Calcium 45 (1) (2009) 38–54.
[43] K. Quick, J. Zhao, N. Eijkelkamp, J.E. Linley, F. Rugiero, J.J. Cox, R. Raouf, M. Gringhuis, J.E. Sexton, J. Abramowitz, R. Taylor, A. Forge, J. Ashmore, N. Kirkwood, C.J. Kros, G.P. Richardson, M. Freichel, V. Flockerzi, L. Birnbaumer, J.N. Wood, TRPC3 and TRPC6 are essential for normal mechanotransduction in subsets of sensory neurons and cochlear hair cells, Open Biol. 2 (5) (2012) 120068.
[44] A.L. Gonzales, Y. Yang, M.N. Sullivan, L. Sanders, F. Dabertrand, D.C. Hill-Eubanks, M.T. Nelson, S. Earley, A PLCγ1-dependent, force-sensitive signaling network in the myogenic constriction of cerebral arteries, Sci. Signal. 7 (327) (2014) ra49.
[45] J. Vassiliadis, C. Bracken, D. Matthews, S. O'Brien, S. Schiavi, S. Wawersik, Calcium mediates glomerular filtration through calcineurin and mTORC2/Akt signaling, J. Am. Soc. Nephrol. 22 (8) (2011) 1453–1461.
[46] S. Devi, A. Li, C.L. Westhorpe, C.Y. Lo, L.D. Abeynaike, S.L. Snelgrove, P. Hall, J.D. Ooi, C.G. Sobey, A.R. Kitching, M.J. Hickey, Multiphoton imaging reveals a new leukocyte recruitment paradigm in the glomerulus, Nat. Med. 19 (1) (2013) 107–112.
[47] W. Kriz, K.V. Lemley, A potential role for mechanical forces in the detachment of podocytes and the progression of CKD, J. Am. Soc. Nephrol. 26 (2) (2015) 258–269.
[48] A.A. Eddy, J.M. López-Guisa, D.M. Okamura, I. Yamaguchi, Investigating mechanisms of chronic kidney disease in mouse models, Pediatr. Nephrol. 27 (8) (2012) 1233–1247.
[49] N. Ramkumar, Y. Gao, D.E. Kohan, Characterization of flow-regulated cortical collecting duct endothelin-1 production, Physiol. Rep. 5 (4) (2017) e13126 (pii).
[50] S. Shen, Y. Jin, W. Li, X. Liu, T. Zhang, W. Xia, Y. Wang, K. Ma, Recombinant human erythropoietin pretreatment attenuates acute renal tubular injury against ischemia-reperfusion by restoring transient receptor potential channel-6 expression and function in collecting ducts, Crit. Care Med. 42 (10) (2014) e663–e672.
[51] Y. Fu, C. Wang, D. Zhang, Y. Xin, J. Li, Y. Zhang, X. Chu, Increased TRPC6 expression is associated with tubular epithelial cell proliferation and inflammation in diabetic nephropathy, Mol. Immunol. 94 (2018) 75–81.