Blockade of the natriuretic peptide clearance receptor attenuates proteinuria in a mouse model of focal segmental glomerulosclerosis

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Abstract
Glomerular podocytes play a key role in proteinuric diseases. Accumulating evidence suggests that cGMP signaling has podocyte protective effects. The major source of cGMP generation in podocytes is natriuretic peptides. The natriuretic peptide clearance receptor (NPRC) binds and degrades natriuretic peptides. As a result, NPRC inhibits natriuretic peptide-induced cGMP generation. To enhance cGMP generation in podocytes, we blocked natriuretic peptide clearance using the specific NPRC ligand ANP(4-23). We then studied the effects of NPRC blockade in both cultured podocytes and in a mouse transgenic (TG) model of focal segmental glomerulosclerosis (FSGS) created in our laboratory. In this model, a single dose of the podocyte toxin puromycin aminonucleoside (PAN) causes robust albuminuria in TG mice, but only mild disease in non-TG animals. We found that natriuretic peptides protected cultured podocytes from PAN-induced apoptosis, and that ANP(4-23) enhanced natriuretic peptide-induced cGMP generation in vivo. PAN-induced heavy proteinuria in vehicle-treated TG mice, and this increase in albuminuria was reduced by treatment with ANP(4-23). Treatment with ANP(4-23) also reduced the number of mice with glomerular injury and enhanced urinary cGMP excretion, but these differences were not statistically significant. Systolic BP was similar in vehicle and ANP(4-23)-treated mice. These data suggest that: 1. Pharmacologic blockade of NPRC may be useful for treating glomerular diseases such as FSGS, and 2. Treatment outcomes might be improved by optimizing NPRC blockade to inhibit natriuretic peptide clearance more effectively.

KEYWORDS
cell signaling, focal segmental glomerulosclerosis, glomerular podocyte, natriuretic peptides
1 | INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is characterized by segmental scarring of the glomerulus, nephrotic syndrome, and often rapid progression to end stage kidney disease (ESKD; D’Agati et al., 2011). The incidence of FSGS has increased ~10-fold in the last 2–3 decades (Kitiyakara et al., 2004; Korbet, 2012), and the disease is now the most common primary glomerular disease causing ESKD in the United States (Kitiyakara et al., 2004; Korbet, 2012). Current therapies for FSGS focus on controlling hypertension and reducing proteinuria by blockade of the renin–angiotensin system (Korbet, 2012). Immunosuppressive therapy is often added based on the rationale that the disease is caused by a dysregulated immune system (D’Agati et al., 2011; Korbet, 2012). Despite these therapies, ~50% of nephrotic patients progress to ESKD over a decade (Korbet, 2012). As a result, there is much interest in developing new therapies.

Glomerular podocytes play a pivotal role in FSGS and other proteinuric kidney diseases (D’Agati et al., 2011; Wiggins, 2007; Wolf et al., 2005). These cells cover the external surface of the glomerular basement membrane and maintain the structural and functional integrity of the glomerular filter (Wiggins, 2007; Wolf et al., 2005). While FSGS is caused by multiple, distinct etiologies (Rosenberg & Kopp, 2017), podocyte injury is a characteristic feature of the disease process, which leads to a decrease in podocyte number (D’Agati et al., 2011; Wiggins, 2007). Because podocytes are terminally differentiated, postmitotic cells, podocytes that are lost cannot be effectively replaced, which causes instability and collapse of the glomerular tuft, glomerulosclerosis, and disease progression (D’Agati et al., 2011; Wiggins, 2007).

Accumulating evidence suggests that cGMP signaling has podocyte protective effects in kidney diseases (Makinoh et al., 2006; Ogawa et al., 2012; Shen et al., 2016; Staffel et al., 2017; Suganami et al., 2001). The beneficial actions of cGMP are mediated, in part, by inhibiting TGFβ, Rho GTPases, and calcium signaling (Francis et al., 2010; Shen et al., 2016). These signaling pathways promote podocyte loss by inducing detachment and apoptosis (Robins et al., 2017; Wang et al., 2012; Zhu et al., 2011). Thus, inhibition of these signaling pathways by cGMP may attenuate the decrease in podocyte number characteristic of FSGS.

Natriuretic peptides potently stimulate cGMP generation in cultured podocytes (Lewko et al., 2004; Theilig et al., 2001). The family of natriuretic peptides includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Potter et al., 2006). ANP and BNP are predominantly produced by the heart, and circulate to act on target organs such as the kidney (Potter et al., 2006). CNP is widely expressed in multiple tissues and cell types including the kidney (Cataliotti et al., 2002; Potter et al., 2006); thus, providing a local source of natriuretic peptides.

Natriuretic peptides stimulate cGMP production by binding to cell surface natriuretic peptide receptors (NPRs) (Pandey, 2014; Potter et al., 2006). ANP and BNP bind to NPRA, and CNP binds to NPRB (Pandey, 2014; Potter et al., 2006). In contrast, the natriuretic clearance receptor natriuretic peptide clearance receptor (NPRC) binds and degrades ANP, BNP, and CNP and negatively regulates the effects of natriuretic peptides (Pandey, 2014; Potter et al., 2006). Podocytes express all three NPRs (Lewko et al., 2004; Potter et al., 2006; Staffel et al., 2017). Moreover, NPRC is highly expressed in glomerular podocytes compared to other glomerular and kidney cell types (Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019). Based on these data, we hypothesized that pharmacologic inhibition of NPRC clearance would enhance local natriuretic peptide levels, promote cGMP signaling in podocytes and, in turn, attenuate glomerular injury. To test this hypothesis, we blocked natriuretic peptide clearance using the specific NPRC ligand ANP(4-23) (Anand-Srivastava et al., 1990; Li et al., 2014; Maack et al., 1987; Nishizawa et al., 2017; Veale et al., 2000) We found that NPRC blockade significantly reduced albuminuria in a mouse model of FSGS.

2 | METHODS

2.1 | Materials

Primary antibodies used for the studies included: (1) A mouse monoclonal antibody to α-smooth muscle actin (Durand-Arczynska et al., 1993) (clone 1A4, catalog number: A5228, Sigma-Aldrich), (2) A mouse monoclonal antibody to actin (Lessard, 1988) (clone C4, catalog number: MA1501, Sigma-Aldrich), (3) Goat polyclonal antibodies to nephrin (Wong et al., 2018) (catalog number: AF3159), (4) A mouse monoclonal antibody to nephrin (clone OTI4H1, catalog number: TA501044, Origene Technologies). Secondary antibodies used for the studies included: (1) A mouse anti-goat polyclonal antibody (catalog number: sc-2354, Santa Cruz Biotechnology), and (2) An anti-mouse polyclonal antibody (catalog number: 7076, Cell Signaling Technology). Additional materials included: ANP(4-23) (GenScript), human ANP, human CNP and PF-04449743 (Sigma-Aldrich), AP-811 (Tocris Bioscience), and LBQ657 and tadalafil (Cayman Chemical).
2.2 | Animal experiments

All experiments were performed using mouse strains on the FVB/NJ background and a transgenic (TG) mouse model of FSGS developed in our laboratory (Wang et al., 2015). These TG mice express a constitutively active Gq α-subunit, which is specifically induced in podocytes with doxycycline (2 mg/ml in drinking water with 2% sucrose to enhance palatability; Wang et al., 2015). In these TG mice treatment with the podocyte toxin puromycin aminonucleoside (PAN) causes robust albuminuria and glomerulosclerosis in TG mice, but only mild disease in non-TG animals.

For the experiments, age and sex-matched male and female mice were used for the studies because the phenotype is similar in both sexes (Wang et al., 2015). Mice were studied at 3–4 months of age and all mice were born within a 5-week period. Baseline 24-h urine collections were collected using metabolic cages specifically designed for collection of mouse urine (Hatteras Instruments). After collecting baseline urines, mice were treated with doxycycline for 1 week and then nephrosis was induced by a single injection of PAN (500 mg/kg) by intraperitoneal (IP) injection and doxycycline was continued an additional 2 weeks. During the 2-week study period, TG and non-TG were treated with the either 10 nmol/kg ANP(4-23) or vehicle (sterile saline) by IP injection 5 days per week (Monday through Friday). Systolic blood pressure was measured as described (Wang et al., 2017) the second week after the PAN injection, following acclimation to the experimental procedure the prior week. Repeat 24-hour urine collections were obtained at day 10 and day 14 following the PAN injection. Mice were euthanized after the last urine collection by injecting 250 mg/kg pentobarbital, IP, followed by bilateral thoracotomy. Blood was immediately obtained by cardiac puncture after euthanasia and then kidneys were removed, weighed and kidney tissue was saved for additional studies as described below. The experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Duke and Durham VA Medical Centers’ Institutional Animal Care and Use Committees. A schematic of the experimental protocol is provided in Figure S1.

To assess the effectiveness of ANP(4-23) at enhancing cGMP generation in wild type mice, we performed a pilot study by treating mice with a single injection of ANP(4-23) (10 nmol/kg by IP injection) or vehicle (normal saline). Urines were then collected in metabolic cages, during the day after injection, overnight, and the following morning during the time periods indicated in Figure 2. Data were expressed as nanograms/milligram creatinine.

2.3 | BP measurements

Systolic BP was measured using a computerized tail-cuff system (Hatteras Instruments) in conscious mice as previously described (Wang et al., 2017). To reduce variability in the results, mice were acclimated to the experimental conditions for a week prior to the BP measurements. This technique has previously been shown to correlate closely with intra-arterial measurements (Whitehall, et al., 2004).

2.4 | Histopathology

After fixation in formalin, light microscopic sections were stained with hematoxylin and eosin (H&E) and Masson trichrome. The slides were evaluated by a pathologist (A.F.B.) blinded to genotype. Each tissue section had more than 20 glomeruli for evaluation. Tubules were examined for tubule dilation and casts, and tubulointerstitial areas were examined for inflammation with or without interstitial fibrosis. There is little tubulointerstitial fibrosis observed in this model over a 14-day period, and this score was based on the severity of inflammation as described below. Abnormalities were graded using a semiquantitative scale of 0–3 (0-normal, 1-mild, 2-moderate, 3-severe) as previously described; Wang et al., 2015; Wang et al., 2019) based on the following criteria:

- **2.4.1 | Glomerulosclerosis**
  1. Normal (baseline): None.
  2. Mild: <10% of glomeruli.
  3. Moderate: 10%–25% of glomeruli.
  4. Severe: >25% of glomeruli.

- **2.4.2 | Tubule injury**
  1. Normal (baseline): None to minimal tubular dilation and casts without tubular degeneration or regeneration.
  2. Mild: Tubular degeneration and regeneration with/without tubular dilation and casts, involving <10% of cortex.
  3. Moderate: Tubular degeneration and regeneration with/without tubular dilation and casts, involving 10–25% of cortex.
  4. Severe: Tubular degeneration and regeneration with/without tubular dilation and casts, involving >25% of cortex.

- **2.4.3 | Tubulointerstitial inflammation**
  1. Normal (baseline): One focus of inflammation (up to 15 mononuclear cells) with or without interstitial fibrosis.
2. Mild: Two foci of 15+ mononuclear cells or 1 focus of 30+ mononuclear cells involving up to 5% of area of cortical parenchyma with or without interstitial fibrosis.

3. Moderate: Inflammation involving >5%–25% of the cortical parenchyma with or without interstitial fibrosis.

4. Severe: Inflammation involving >25% of the cortical parenchyma with or without interstitial fibrosis.

2.5 Cell culture studies

The immortalized mouse podocyte cell line (Schwartz et al., 2001) was a gift of Dr. Paul E. Klotman (Mount Sinai Medical Center, New York, NY). The cells were derived from animals bred with the H-2Kb-tsA58 Immortomice (Charles River Laboratories). Podocytes were selected for expression of the podocyte markers WT-1, synaptopodin, and podocalyxin. To permit immortalized growth, cells were grown at 33°C in medium supplemented with 100 units/ml gamma-interferon to induce the H-2Kb-tsa58 promoter driving synthesis of the temperature sensitive (tsA58) SV-40 T antigen (permissive conditions). For differentiation, cells were grown at 37°C in medium lacking gamma-interferon, resulting in degradation of the T antigen (non-permissive conditions). Our lab further characterized differentiated cells by RT-PCR and they also express low levels of the podocyte proteins nephrin and podocin. For the experiments, the immortalized mouse podocyte cell line was maintained in culture and were plated in either 6- or 12-well tissue culture clusters (Corning-Costar) and differentiated for ~7 days prior to study as previously described (Flannery & Spurney, 2006).

For the cGMP studies, podocytes were made quiescent by incubation in serum free medium overnight. Cells were then treated with vehicle or the following concentrations of compounds in the presence of the following compounds: 5 µM PF-04497943, 5 µM tadalafil, 10 µM LBQ657, 1 µM ANP(4-23), 100 µM SNAP, 1 µM CNP, and either 0.1 nM, 1 nM, 10 nM, or 1 µM ANP as indicated. Additional ANP or CNP was added to the medium in the afternoon prior to study to supplement any ANP which may have been degraded during the incubation period. The following morning, cells were harvested and apoptotic cells were identified by annexin V staining using kits from BD Pharmingen according to the directions of the manufacturer. Quantitation of the apoptotic cells was performed by flow cytometric analysis at the Duke Comprehensive Cancer facility. For the annexin V studies, apoptotic podocytes were differentiated from necrotic cells by staining with 7-amino-actinomycin D. For evaluation of these data, basal levels of apoptosis (cells treated with 0.1% FBS and vehicle) were subtracted from the results, and data expressed as the percent apoptosis above basal.

2.6 Expression of glomerular mRNAs

Reverse transcription (RT) followed by a quantitative polymerase chain reaction (Q-RT-PCR) was performed using an iCycler TM (Bio-Rad Laboratories, Inc.). For the studies, total cellular RNA was prepared using glomerular preparations and the Trizol reagent according to the manufacturer's directions (Life Technologies Inc.). The RNA was treated with RNAase free DNAase (Qiagen) and then reverse-transcribed with Superscript reverse transcriptase (Invitrogen) and oligo (dT) primers. Real-time quantitative PCR was performed using an iCycler Q-PCR machine and the universal SYBR Green PCR Master Mix Kit (Perkin-Elmer). The amplification signals were normalized to the endogenous cyclophilin mRNA level. The primer sequences used for Q-RT-PCR were as follows: CNP forward: 5′-AAT ACA AAG GCG GCA ACA AG—3′ & reverse, 5′—TAA CAT CCC AGA CCG CTC AT—3′. NPRC forward, 5′—AGC TGG CTA CAG CAA GAA GG—3′ & reverse, 5′—CGG CGA TAC CTT CAA ATG TC—3′, Nephrilysin forward, 5′—CCA AAC TTA AGC CTA TTC TTA C—3′ & reverse, 5′—CCA TTA TGA ACC TCC AGG AC—3′, PDE5a forward, 5′—AGA CAT GG TCA ACG CAT GTT T—3′ & reverse, 5′—TAT GGG CTC GGA TGC CTT C—3′, PDE9a forward, 5′—ACC TGT TCT GTA TCG CCA CC—3′ & reverse, 5′—CTT CAC AGC CAC AGG TCT CA—3′, Cyclophilin, forward, 5′—GGC CGA TGA CGA GCC C—3′ & reverse, 5′—TGT TCT TGG AAC TTT GTC TGC AA—3′, Fibronectin forward 5′—CGA GTT GAC AGA GAC CAC AA—3′ & reverse, 5′—CTG GAG TCA AGC CAG ACA CA—3′; collagen type 1, α1 (COL1A1) forward 5′—ATC TCC TGG TGC TGA TGG AC—3′ & reverse 5′—ACC TGG TTT GCC AGG TAC AC—3′, α-smooth muscle actin (SMA) forward 5′—GAG GCA CCA CTG ACC CCT AA—3′ & reverse 5′—CAT TCT CAG AGT CCA GCA CA—3′.
2.7 Statistical analysis

Data are presented as the mean ± standard error of the mean and statistical analyses were performed using the Prism computer program (GraphPad Software, Inc.). For comparison of two groups of continuous variables, data were analyzed by a t-test. For comparison of more than 2 groups of continuous variables, data were analyzed by either: (1) A one-way or two-way analysis of variance (ANOVA) as specified by the Prism program, followed by Sidak’s multiple comparisons post-test. (2) A Kruskal–Wallis test followed by Dunn’s multiple comparison post-test, or (3) An ANOVA followed by Dunnett’s multiple comparisons post-test. For non-continuous variables (histopathology), data were analyzed using a Fisher’s exact test using the number mice with the specified histologic abnormality. Graphs of the histologic findings were presented as the percentage of mice with the specified abnormality to permit a more effective comparison of the differences between the experimental groups in studies with an imbalance in the number of mice in each group.

3 RESULTS

3.1 Effect of natriuretic peptides on podocyte cGMP generation and apoptosis

Generation of cGMP is stimulated by nitric oxide and by natriuretic peptides (Francis et al., 2010). Natriuretic peptides potently stimulate cGMP generation in cultured podocytes; whereas, the effects of nitric oxide on podocyte cGMP generation are of a lesser magnitude (Lewko et al., 2004, 2006; Theilig et al., 2001). As shown in Figure 1a, we measured podocyte cGMP generation in the presence or absence of ANP, CNP, and the nitric oxide donor SNAP (S-nitroso-N-acetyl-DL-penicillamine). Both ANP and CNP potently stimulated cGMP generation in cultured podocytes. In contrast, stimulation of podocytes with SNAP-induced little cGMP generation. We next determined if ANP protected glomerular podocytes from apoptosis induced by PAN. As shown in Figure 1b, treatment with PAN increased apoptosis above basal levels, and this increase in apoptosis was significantly inhibited by treatment with either ANP or CNP. Taken together with published studies (Lewko et al., 2006; Staffel et al., 2017; Theilig et al., 2001), these data suggest that: (1) ANP and CNP potently stimulate cGMP generation in podocytes, (2) ANP and CNP have podocyte protective effects in vitro, and (3) Given that ANP stimulates NPRA and CNP stimulates NPRB, podocytes express both NPRA and NPRB.

In addition to expressing NPRA and NPRB, podocytes express high levels of the NPRC (Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019), which binds and degrades natriuretic peptides (Potter et al., 2006). Natriuretic peptide-induced cGMP generation is also negatively regulated by additional mechanisms including phosphodiesterases (PDEs) and the neutral endopeptidase (neprilysin) (Potter et al., 2006). The family of PDE isoenzymes hydrolyze both cAMP and/or cGMP within the cell, and these enzymes are found in multiple tissues and cell types (Dousa, 1999; Maurice et al., 2014). However, only PDE5 and PDE9 are selective for cGMP, and are expressed in kidney (Dousa, 1999; Maurice et al., 2014). Neprilysin cleaves and inactivates circulating natriuretic peptides and the enzyme is also widely expressed (Potter et al., 2006). Moreover, high levels of the enzyme are expressed in podocytes (Debiec et al., 2002; Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019). As shown in Figure 1c,d, mRNA for NPRC, neprilysin, PDE5, and PDE9 are expressed in cultured podocytes and both NPRC and neprilysin are highly expressed at both the mRNA and protein level. Thus, multiple mechanisms likely regulate natriuretic peptide responsiveness in glomerular podocytes.

To examine the most effective strategy to enhance natriuretic peptide-induced cGMP generation in podocytes, we measured ANP-induced cGMP generation in the presence of the ANP analog ANP(4-23), which specifically blocks binding of natriuretic peptides to NPRC without affecting NP binding to either NPRA or NPRB (Maack et al., 1987; Nishizawa et al., 2017; Veale et al., 2000; William et al., 2008). We compared the effects of ANP(4-23) with the neprilysin inhibitor LBQ657 (Campbell, 2017), the specific PDE5 inhibitor tadalafil (Rotella, 2002) the specific PDE9 inhibitor PF-0449613 (Lee et al., 2015) at doses that effectively blocked either NPRC, neprilysin, PDE5, or PDE9. As shown in Figure 1e, blockade of NPRC-enhanced cGMP generation at low nanomolar concentrations of ANP; whereas neprilysin inhibition and inhibition of either PDE5 or PDE9 had little effect on cGMP generation at the concentrations of ANP used in the experiments.

3.2 Effect of ANP (4-23) on urinary cGMP generation in wild type mice

Based on these findings in cultured podocytes, we hypothesized that augmenting natriuretic peptide signaling by blockade of NPRC might enhance the podocyte protective actions of endogenous natriuretic peptides in vivo. To investigate this possibility, we evaluated the effect of ANP (4-23) in wild type mice. For the studies, urinary cGMP excretion was measured after a single, IP injection of ANP (4-23) as shown in Figure 2. Urine was collected at the indicated times over a 24-hour period (Figure 2a, period 1, 2, and 3). There was a significant increase in urinary
FIGURE 1  Effect of natriuretic peptides on podocyte cGMP generation and apoptosis. (A) Both ANP (1 µM) and CNP (1 µM) potently stimulated cGMP generation. In contrast, SNAP (100 µM)-induced little cGMP generation. (b) PAN significantly enhanced apoptosis and this increase in apoptosis was inhibited by ANP and CNP. (c & d) Podocyte express mRNA for NPRC, neprilysin, PDE5, and PDE9. NPRC and neprilysin were highly expressed in cultured podocytes at both the mRNA and protein level. (e) Podocytes were treated with the indicated doses of ANP in the presence or absence of the NPRC ligand ANP(4-23) (1 µM), the neprilysin inhibitor LBQ657 (10 µM), the PDE5 inhibitor tadalafil (5 µM) or the PDE9 inhibitor PF-0449613 (5 µM). Pharmacologic blockade of NPRC was the most effective strategy to potentiate natriuretic peptide-induced cGMP generation. Four to six tissue culture wells were studied in each group. \( p < 0.01 \) or \( p < 0.001 \) versus vehicle by ANOVA followed by Sidak’s multiple comparisons post-test, *\( p < 0.05 \) versus PDE5 by Kruskal–Wallis test followed by Dunn’s multiple comparisons test, \( p < 0.01 \) versus PAN or \( p < 0.001 \) versus basal by an ANOVA followed by Sidak’s multiple comparisons post-test, **\( p < 0.01 \) versus LBQ657 or \( p < 0.001 \) versus tadalafil, or PF4449613 by ANOVA followed by Sidak’s multiple comparisons post-test.

FIGURE 2  Effect of ANP(4-23) on urinary cGMP excretion in non-TG wild type mice. (a) Mice were given a single, IP injection of 10 nmol/kg ANP(4-23) or vehicle at the beginning of period 1 (11AM). Urine was then collected during periods 1, 2, and 3 at the indicated times. (b) Urinary cGMP excretion was significantly increased in period 2, and the increase in urinary cGMP excretion was sustained in period 3. (c) Urine output increased significantly during period 2 and returned to baseline in period 3. Four non-TG mice were studied in each group. *\( p < 0.05 \) versus vehicle by ANOVA followed by Dunnett’s multiple comparisons post-test. Data in (c) were divided by time (h) to correct for the different durations of the urine collections. Note that the y-axis of (a) is logarithmic.
cGMP excretion in wild type mice during the overnight collection that was sustained in the urine collected the following day (Figure 2b). Moreover, urine output increased significantly during the nighttime urine collection (Figure 2c).

3.3 | Effect of ANP(4-23) on kidney disease in TG mice

We next evaluated the effects of ANP(4-23) on kidney disease in TG mice (Wang et al., 2015) using the protocol described in Section 2 (see also the schematic in Figure S1). This TG model expresses a doxycycline inducible, constitutively active Gqα-subunit specifically in podocytes (Wang et al., 2015). In these animals, treatment with a single dose of the podocyte toxin PAN causes robust albuminuria in TG mice, but only mild disease in non-TG animals. For the experiments, we chose a 10 nmol/kg dosage of ANP(4-23) that had previously been demonstrated to reduce systemic blood pressure in spontaneously hypertensive rats but not in control Wistar-Kyoto rats (Li et al., 2014). Figure 3 shows the effects of ANP(4-23) on albuminuria, glomerular histology, and systemic blood pressure. We found that PAN-induced heavy proteinuria at day 14 in TG mice treated with vehicle (normal saline) compared to baseline albuminuria (Figure 3a). This increase in albuminuria at day 14 was significantly reduced by treatment with ANP(4-23) (Figure 3a). PAN had little effect on albuminuria in non-TG mice (Table 1). Treatment with ANP(4-23) also reduced glomerular injury (Figure 3b–f), but this difference was not statistically significant. A similar pattern was seen in tubulointerstitial inflammation and tubular dilation and casts (Table 2). Systolic BP was similar in TG mice receiving ANP(4-23) and in the vehicle-treated group (Figure 3g). There was no clear relationship between the severity of glomerular injury and systolic BP (Figure S2). A subgroup analysis of mice with severe glomerulosclerosis found a systolic BP of 127.5 ± 2.3 mmHg (N = 4) in the TG mice treated with vehicle compared to a systolic BP of 132 ± 5.7 mmHg (N = 5; p = NS) in the ANP(4-23)-treated TG mice.

3.4 | Effect of male and female sex on the experimental outcomes

To examine the sex-dependent effects of the treatment, albuminuria, and glomerular histologic findings are presented for male and female mice in Figure 4. As shown

![Figure 3](image_url)

**FIGURE 3** Effect of ANP(4-23) on kidney disease in TG mice. (a) PAN-induced heavy proteinuria at day 14 in TG mice treated with vehicle compared to baseline albuminuria. The increase in albuminuria at day 14 was significantly reduced by treatment with ANP(4-23). (b–f) Treatment with ANP(4-23) reduced the number of mice with glomerular injury but this difference was not statistically significant. (g) Systolic BP was similar in TG mice treated with vehicle and ANP(4-23). Ten to 13 TG mice were studied in each group. **p = 0.0002 versus baseline or †p = 0.003 versus vehicle-treated TG mice at day 14 by ANOVA followed by Sidak’s multiple comparisons post-test. Kidney sections were stained with Masson’s trichrome stain.
in Figure 4a, albuminuria was significantly increased in female mice on day 14 and this increase in albuminuria was significantly reduced by treatment with ANP (4-23). A similar pattern was seen in male mice, but the differences were not statistically significant. There was a significant effect of treatment with ANP(4-23) on albuminuria ($p = 0.0016$) by a two-way analysis of variance (ANOVA), but there was no significant effect of sex on the results. Consistent with the albuminuria, the severity of glomerulosclerosis was more severe in female mice compared to male animals in both the vehicle-treated group (Figure 3b) and the ANP-(4-23) treated group (Figure 4c), but these differences were not statistically significant. Lastly, there was no significant difference in glomerulosclerosis by sex in mice treated with ANP(4-23) compared to mice treated with vehicle (compare Figure 4b,c).

We next evaluated the effect of ANP(4-23) treatment on expression of podocyte protein mRNAs (nephrin, podocin, Wilms tumor 1, or WT-1), markers of fibrosis (fibronectin, collagen type 1, α1, or COL1A1) and a marker myofibroblast differentiation (α-smooth muscle actin or α-SMA) (Davis et al., 2012; Tomasek et al., 2002). In these studies, mRNA levels of these transcripts were similar in the ANP(4-23)- and vehicle-treated non-TG groups and these data were combined for the analyses. As shown in Table 3, there was no significant differences in expression of mRNAs for nephrin, podocin, and WT-1 in both TG groups compared to non-TG control mice. In contrast, markers of fibrosis and myofibroblast differentiation were increased in both groups of TG mice compared to non-TG controls, and these differences were statistically significant for fibronectin and COL1A1.

### 3.5 Effect of ANP(4-23) on glomerular podocytes and kidney fibrosis

Figure 5a–c show the effects of ANP(4-23) on expression of nephrin and α-SMA proteins in mouse glomerular preparations. As shown in Figure 5a,b, nephrin levels were better preserved in ANP(4-23)-treated TG mice compared to TG mice treated with vehicle. Consistent with the mRNA studies (Table 3), α-SMA protein levels were significantly increased in glomerular preparations from TG mice treated with either vehicle or ANP(4-23) (Figure 4a,c). A similar pattern was observed in collagen expression in kidney cortex by Sirius red staining in TG mice compared to non-TG mice (Figure 5d) but this difference was not significant.

### Table 1 Albuminuria (µg/mg creatinine) in non-TG controls

|          | Vehicle   | ANP(4-23) |
|----------|-----------|-----------|
| Baseline | 11.8 ± 0.8| 13.7 ± 1.7|
| 10 days  | 9.9 ± 1.5 | 12.8 ± 1.7|
| 14 days  | 16.9 ± 4.0| 9.0 ± 1.4 |

*Note: Data are expressed as the mean ± SEM. Number of mice: Vehicle = 6, ANP(4-23) = 7.*

### Table 2 Histopathologic findings (number of mice)

|                  | Glomerulosclerosis | Tubular dilation & casts | Tubulointerstitial inflammation |
|------------------|--------------------|--------------------------|---------------------------------|
| TG vehicle (N = 11) | None 3            | None 2                   | None 2                          |
|                  | Mild 2            | Mild 6                   | Mild 5                          |
|                  | Moderate 2        | Moderate 3               | Moderate 4                      |
|                  | Severe 4          | Severe 0                 | Severe 0                        |
| TG ANP(4-23) (N = 11) | None 5          | None 3                   | None 4                          |
|                  | Mild 1            | Mild 7                   | Mild 2                          |
|                  | Moderate 0        | Moderate 1               | Moderate 5                      |
|                  | Severe 5          | Severe 0                 | Severe 0                        |
| Control Vehicle (N = 5) | None 6         | None 6                   | None 6                          |
|                  | Mild 0            | Mild 0                   | Mild 0                          |
|                  | Moderate 0        | Moderate 0               | Moderate 0                      |
|                  | Severe 0          | Severe 0                 | Severe 0                        |
| Control ANP(4-23) (N = 5) | None 6         | None 6                   | None 6                          |
|                  | Mild 0            | Mild 0                   | Mild 0                          |
|                  | Moderate 0        | Moderate 0               | Moderate 0                      |
|                  | Severe 0          | Severe 0                 | Severe 0                        |

*Note: During the study, two female TG mice died in the vehicle group and two TG mice (1 male and 1 female) died in the ANP(4-23) group.*
There were no significant differences in podocyte numbers in the TG and non-TG animals (Figure 5e).

### 3.6 Effect of ANP(4-23) on urinary cGMP excretion in TG and non-TG mice

Lastly, we measured urinary cGMP excretion to assess the effectiveness of NPRC blockade. There was no significant difference in urinary cGMP excretion in TG mice at baseline. Treatment with PAN-enhanced urinary cGMP excretion in all groups of mice compared to the baseline measurements. This increase in urinary cGMP excretion was statistically significant compared to baseline in TG mice treated with either vehicle or ANP(4-23) excretion (Figure 6). There was also an increase in urinary cGMP excretion in ANP(4-23)-treated TG mice compared to the vehicle-treated TG animals, but this difference was not statistically significant after correcting for multiple comparisons.
DISCUSSION

In this study, we found that: (1) Both ANP and CNP protected cultured podocytes from PAN-induced apoptosis. (2) Pharmacologic blockade of NPRC was more effective at enhancing podocyte cGMP generation compared to either nepri-lysin inhibition or inhibition of either PDE5 or PDE9. (3) Blockade of NPRC in vivo inhibited albuminuria and improved expression of the podocyte marker nephrin in a mouse model of FSGS. (4) The beneficial effects of pharmacologic NPRC blockade were accomplished without a significant change in systemic blood pressure, and (5) There was a trend toward improvement in the histopathologic features of the disease but markers of glomerular fibrosis and myofibroblast differentiation were not significantly affected by the treatment strategy. Treatment with ANP(4-23) increased urinary cGMP excretion in TG mice compared vehicle-treated TG mice, but this difference was not statistically significant. Given that ANP(4-23) is rapidly degraded in mouse serum (Nishizawa et al., 2017), we posit that more effective pharmacologic inhibition of NP clearance may further enhance the beneficial effects of the treatment strategy.

Multiple mechanisms negatively regulate the biological effects of natriuretic peptides including NPRC, nepri-lysin, and PDEs such as the cGMP-specific PDEs including PDE5 and PDE9 (Potter et al., 2006; Rotella, 2002). All these mechanisms likely play a role in regulating the biological effects of natriuretic peptides in glomerular podocytes. Both NPRC and nepri-lysin compete for extracellular NP ligands; whereas, PDEs act intracellularly to hydrolyze ATP and/or cGMP. Cultured podocytes expressed high levels of both NPRC and nepri-lysin, consistent with published studies (Debiec et al., 2002; Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019). Moreover, both NPRC and nepri-lysin play important roles in removing natriuretic peptides from the circulation and inhibiting their activity (Potter et al., 2006). Blockade of NPRC was, however,
the most effective approach for augmenting ANP-induced cGMP generation in cultured podocytes compared to inhibition of either neprilysin, PDE5, or PDE9 inhibition (Figure 1e). This finding is consistent with published studies using pharmacologic inhibitors of NPRC or neprilysin in vivo (Potter, 2011). As a result, infusion of NPRC antagonists enhances both ANP concentrations and the physiologic effects of natriuretic peptides to a greater extent than pharmacologic neprilysin inhibition (Charles et al., 1996; Hashimoto et al., 1994; Kukkonen et al., 1992; Okolicany et al., 1992; Potter, 2011). The potent ability of NPRC blockade to enhance cGMP generation in cultured podocytes may be due to: (1) PDEs hydrolyze cGMP inside the cell and inhibition is effective only if significant intracellular cGMP is generated, (2) The high levels of NPRC expressed by podocytes (Debiec et al., 2002; Park et al., 2018; Staffel et al., 2017; Wilson et al., 2019), (3) The high affinity of NPRC for natriuretic peptides (10–140 pM) compared to the lower affinity of neprilysin (Km ~10 to 100 µM) (Hubers & Brown, 2016; Potter et al., 2006). Given the low concentrations of natriuretic peptides in biological fluids (1–100 pM) (Potter et al., 2006), NPRC is more effective than neprilysin at scavenging extracellular natriuretic peptides. These differences are an important advantage of NPRC blockade compared to other pharmacologic approaches for enhancing cGMP generation in podocytes.

ANP (4-23) is a specific NPRC ligand with low nanomolar affinity for NPRC (Anand-Srivastava et al., 1990; Li et al., 2014; Maack et al., 1987; Nishizawa et al., 2017; Veale et al., 2000). In these studies, we found that a single, 10 nmol/kg dose of ANP (4-23) (~1.1 mg/kg) significantly enhanced urinary cGMP excretion in non-TG wild type mice (Figure 2). These data suggest that treatment with ANP (4-23) effectively inhibited NP clearance in wild type mice. We did not anticipate the increase in urinary cGMP excretion in all groups of mice treated with PAN (Figure 5). This enhanced cGMP excretion may have hindered detecting a difference in urinary cGMP excretion in TG mice treated with ANP (4-23) compared to vehicle-treated TG mice by adding another variable that affected cGMP generation in vivo and, in turn, increased variability in the data. Alternatively, repeated dosages of the drug may have reduced the effectiveness of the treatment approach (Agvald et al., 1999; Freedman & Lefkowitz.). This desensitization to pharmacologic therapy is characteristic of agonist drug therapy (Agvald et al., 1999; Freedman & Lefkowitz.), which is directly relevant to cGMP signaling (Agvald et al., 1999). In addition to inhibiting NPRC clearance, ANP (4-23) is reported to stimulate intracellular signaling by NPRC including activating phospholipase C, inhibiting cAMP generation via Gi, and inhibiting L-type ion channels (PLC) (El Andalousi et al., 2013; Li et al., 1998; Murthy et al., 1998; Rose & Giles, 2008; William et al., 2008). Thus, the effects of ANP (4-23) might be mediated, at least in part, by stimulating NPRC. Future studies could address this possibility by studying other high affinity, specific NPRC ligands such as AP-811 (Koyama et al., 1994; Nishizawa et al., 2017; Veale et al., 2000; William et al., 2008) which does not act as an agonist at the NPRC receptor (Becker et al., 2014; William et al., 2008) but effectively inhibits NP clearance from the circulation in vivo (Wegner et al., 1995).

The studies used both male and female mice for the experiments because previous studies found no significant difference in the outcomes by sex (Wang et al., 2015). In the present study, we also found no significant difference in albuminuria or glomerular injury in groups of male and female mice treated with either vehicle or ANP (4-23). However, the improvement in albuminuria was predominantly the result of a decrease in albuminuria in the female animals (see Figure 4). While there was a significant effect of treatment [vehicle versus ANP (4-23)] on the experimental outcomes, sex had no significant effect on either albuminuria or glomerulosclerosis using a two-way ANOVA. It is possible that female mice are more
responsive to the benefits of NPRC blockade compared to male mice; however, this possibility will require further study.

In addition to reducing albuminuria in the TG animals, pharmacologic blockade of NPRC improved expression of the podocyte protein nephrin in ANP(4-23)-treated mice compared to TG mice treated with vehicle. This beneficial effect occurred without significantly affecting nephrin mRNA levels in podocytes. Regulation of nephrin expression is complex and includes both transcriptional and post-transcriptional mechanisms (Ren et al., 2005; Ristola et al., 2013; Yamauchi et al., 2006). While we can only speculate on the molecular mechanism(s), nephrin plays a key role in maintaining glomerular filtration barrier integrity (Li et al., 2015). Thus, the beneficial effects of NPRC blockade on podocyte nephrin levels may have contributed to the decrease in albuminuria observed in this study.

In contrast to the beneficial effects of pharmacologic NPRC blockade on albuminuria and nephrin expression, ANP(4-23) had little effect on markers of fibrosis or myofibroblast differentiation. The lack of a beneficial effect on these experimental outcomes may be due to an insufficient drug dosage or desensitization to the effects of the drug as mentioned above. In addition, a subgroup analysis of mice with severe glomerulosclerosis found a trend toward increased systolic BP in ANP(4-23)-treated TG mice compared to vehicle-treated TG mice, which could enhance fibrosis in the ANP(4-23) group. Alternately, increasing the duration of the study may be required to observe a beneficial effect of drug treatment on markers of fibrosis or myofibroblast differentiation. Lastly, pharmacologic potentiation cGMP generation may have effects on renal hemodynamics (Tapia et al., 2012), which may have contributed to the decrease in albuminuria, without affecting glomerulosclerosis.

Lastly, we found that stimulation of either the NPRA or NPRB receptor potently stimulated cGMP generation in cultured podocytes, consistent with a published study (Lewko et al., 2004). In contrast to this previous study (Lewko et al., 2004), however, we found that ANP was a more potent agonist than CNP at similar concentrations (1 µM). While the differing findings may be related to the specific cell lines and/or culture conditions used in the experiments, previous studies suggest: (1) NPRA is highly expressed in glomerular podocytes compared to more modest expression of NPRB (Park et al., 2018), and (2) Activation of NPRB by CNP generally induces cGMP generation of a lower magnitude than activation of NPRA by ANP (Pandey, 2014). These findings may explain the more potent effects on cGMP generation of ANP compared to CNP in the present study.

In summary, NPs protect podocytes from apoptosis. Inhibition of NP clearance is a potent strategy to stimulate NP-induced cGMP generation in both cultured podocytes and in vivo. In a mouse model of FSGS, pharmacologic NPRC blockade decreases albuminuria and preserves podocyte nephrin expression but did not significantly affect the histopathologic features of the disease, systemic blood pressure, or markers of renal fibrosis and myofibroblast differentiation. These data suggest that pharmacologic blockade of NPRC is a promising strategy to treat glomerular diseases. Optimizing the effectiveness of NPRC blockade may further enhance the beneficial effects of this treatment approach.

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