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TITLE: Promoting Podocyte Protective Natriuretic Peptide Signaling in Proteinuric Kidney Diseases

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14. ABSTRACT
Glomerular podocytes play a key role in glomerular disease processes. Accumulating evidence suggests that cGMP signaling has podocyte protective effects in kidney diseases. Production of cGMP is stimulated by nitric oxide and by natriuretic peptides (NPs). NPs are the predominant source of cGMP generation in podocytes. NPs stimulate cGMP production by binding to NP receptors (NPRs). NPRA and NPRB stimulate cGMP generation. In contrast, NPRC binds and degrades NPs. Podocytes express all three NPRs (NPRA, NPRB, and NPRC). We hypothesized that blockade of NPRC would enhance local NP levels, promote cGMP signaling in podocytes and, in turn, attenuate podocyte and glomerular injury. To examine this hypothesis, we blocked clearance of NPs by NPRC using the pharmacologic agent ANP (4-23), which specifically binds NPRC without binding NPRA or NPRB. We then examined the effect of inhibiting NP clearance in a mouse model of focal segmental glomerulosclerosis (FSGS) created in our laboratory. We found that treatment with ANP(4-23) significantly reduced albuminuria (1426 ± 425 [vehicle] vs. 383 ± 157 [ANP(4-23)] ug/mg creatinine; P = 0.003), preserved expression of the podocyte marker nephrin and tended to reduce the number of mice with glomerular injury (83% [vehicle] vs 54% [ANP(4-23)]; P = NS). Systolic BP was similar in mice receiving ANP(4-23) and in the vehicle treated group (129 ± 3 [vehicle] vs. 127 ± 3 [ANP(4-23)] mm Hg; P = NS). Urinary cGMP excretion tended to be higher in ANP(4-23) treated mice (6.7 ± 1.0 ng/mg creatinine) compared to mice treated with vehicle (4.9 ± 1.0 ng/mg creatinine), but this difference was not statistically significant. These data suggest that: 1. Pharmacologic blockade NPRC may be a useful strategy for treating proteinuric kidney diseases, and 2. Treatment outcomes might be improved by optimizing blockade of the NPRC to more effectively inhibit clearance of NPs from the circulation.

15. SUBJECT TERMS
Diabetic kidney disease (DKD), focal segmental glomerulosclerosis (FSGS)

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TABLE OF CONTENTS

10. Introduction

Proteinuric kidney diseases are the most common causes of end stage kidney disease (ESKD) in the US. Two prototypical proteinuric diseases are focal segmental glomerulosclerosis (FSGS) and diabetic kidney disease (DKD). Glomerular podocytes play a key role in the pathogenesis of both FSGS and DKD. Moreover, podocyte damage is exacerbated by the hyperglycemic environment in diabetes. Accumulating evidence suggests that natriuretic peptides (NPs) have direct podocyte protective effects in glomerular disease processes, and improve the diabetic milieu by acting on adipocytes to reduce insulin resistance and decrease hyperglycemia. The effects of NPs are mediated by stimulating cGMP generation by binding to NP receptors (NPRs). NPRA and NPRB stimulate cGMP generation. In contrast, the NPR clearance receptor (NPRC) binds and degrades NPs. All three receptors are present in podocytes and adipocytes, and generation of cGMP is limited by NRPC-mediated clearance of NPs. We hypothesized that blockade of NPRC would enhance local NP levels, promote cGMP signaling in both podocytes and adipocytes, and attenuate glomerular injury in DKD and FSGS. To examine this hypothesis, three specific aims were proposed: 1. Pharmacologic blockade of NPRC in FSGS, 2. Pharmacologic blockade of NPRC in type 2 diabetes, and 3. Podocyte specific knockout (KO) of NPRC in FSGS. To accomplish these goals, we have proposed to study the effects of pharmacologic blockade of NPRC in mouse models of FSGS and type 2 diabetes, as well as the effect of podocyte specific deletion of NPRC in a mouse model of FSGS. Results of these experiments are described below.

11. Keywords:

focal segmental glomerulosclerosis, diabetes mellitus, diabetic kidney disease, diabetic nephropathy, natriuretic peptides, cGMP, cell signaling

12. Accomplishments

Major goals/specific aims of the project:

Specific aim #1: Pharmacologic blockade of NPRC in FSGS.

These studies utilize ANP(4-23), which binds NPRC without binding NPRA or NPRB and, in turn, inhibits NP clearance by NPRC and potentiates the actions of endogenous NPs. To examine the translational therapeutic potential of NPRC blockade in FSGS, the effects of ANP(4-23) were studied in a mouse model of FSGS developed in our laboratory. As shown in Figure 1,

Specific aim #2: Pharmacologic blockade of NPRC in type 2 diabetes.

To determine if ANP(4-23) recapitulates the beneficial metabolic effects of adipocyte specific NPRC KO, we will treat a robust model of type 2 diabetes (BTBR ob/ob mice) with ANP(4-23) and examine the effect on obesity, hyperglycemia and insulin resistance.

Specific aim #3: Podocyte specific KO of NPRC in FSGS.

These studies will create mice with a podocyte specific NPRC knockout (KO). We will then determine if NPRC KO ameliorates FSGS in our mouse model.

Accomplishments toward these goals:

Specific aim #1: To investigate the role of pharmacologic blockade of NPRC in FSGS we inhibited clearance of NPs by NPRC using the pharmacologic agent ANP (4-23), which specifically binds NPRC without binding NPRA or NPRB. For the experiments, we used a mouse transgenic (TG) model of focal segmental glomerulosclerosis (FSGS) created in our laboratory (J Clin Invest 125:1913, 2015). These TG mice express a constitutively active Gq α-subunit specifically in podocytes. In these animals, treatment with a single dose of the podocyte toxin puromycin aminonucleoside (PAN) causes robust albuminuria in TG mice, but only mild disease in non-TG animals. As shown in Figure 1, we found that PAN induced heavy proteinuria at day 14 in TG mice treated with vehicle (phosphate buffered saline or PBS) compared to baseline albuminuria. The increase in albuminuria at day 14 was significantly reduced by treatment with ANP(4-23). Treatment with ANP(4-23) also tended to reduce the number of mice with glomerular injury (Figure 1B-1F). Systolic BP was similar in mice receiving ANP(4-23) and in the vehicle treated group (Figure 1G).

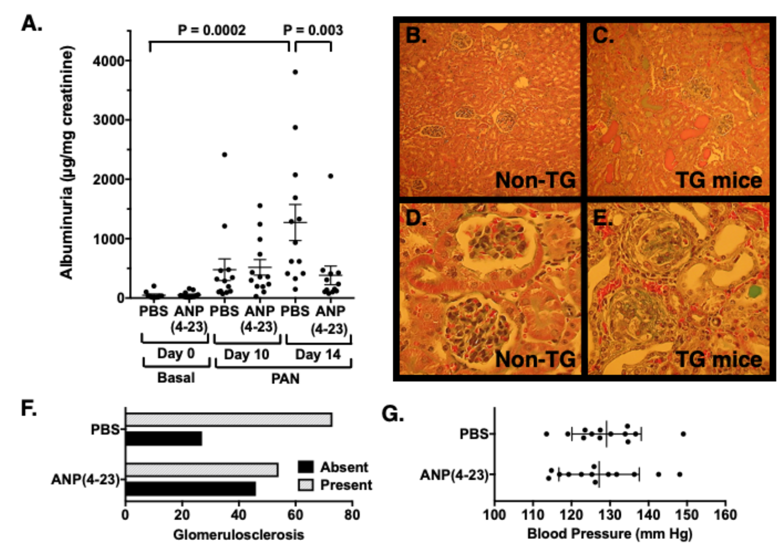

Figure 1. (A)Treatment of TG mice with PAN induces robust albuminuria in TG mice, which was significantly reduced by treatment with ANP(4-23). (B-E) PAN caused severe glomerular and tubular injury in TG mice compared to non-TG control mice treated with PAN. (F) ANP(4-23) tended to reduce glomerular injury in TG mice. (G) Systemic BP was similar in TG mice treated with either saline or PAN. Kidney sections were stained with Masson trichrome. Albuminuria data analyzed by ANOVA followed by Sidak's multiple comparisons test.

We next evaluated the effect of ANP(4-23) treatment on expression of podocyte proteins (nephrin, podocin, Wilms tumor 1 or WT1), and markers of fibrosis (α-smooth muscle actin or α-SMA, fibronectin, collagen type 1, alpha 1 or COL1A1). As shown in Table 1,there was no significant

TABLE 1. Relative expression of mRNA (mean ± SEM)

	Non-TG Controls	TG mice Vehicle	TG mice ANP(4-23)
Nephrin	1.0000 ± 0.5782	0.7501 ± 0.2819	0.7964 ± 0.1905
Podocin	1.0000 ± 0.5880	0.7355 ± 0.6438	0.7683 ± 0.1992
WT1	1.0000 ± 0.3989	1.0630 ± 0.2990	0.9896 ± 0.1789
α-SMA	1.0000 ± 0.3356	2.877 ± 0.6527	2.143 ± 0.5401
Fibronectin	1.0000 ± 0.4904	11.49 ± 4.0060**	10.59 ± 2.425*
COL1A1	1.0000 ± 0.5782	18.44 ± 7.038†	14.18 ± 6.893**

Analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test (†P<0.005, **P<0.01 or *P<0.025 versus non-TG controls) difference in expression podocyte proteins, although nephrin and podocin mRNA levels tended to be decreased compared to non-TG control mice. In contrast, the fibrotic markers were increased similarly in both groups of TG mice compared to non-TG controls, and these differences were statistically significant for fibronectin and type 1 collagen. Consistent with increased fibrosis in both groups of TG mice, α-SMA protein levels was significantly increased in glomerular preparations from TG mice treated with either vehicle or ANP(4-23), as shown in Figure 2A&B. In contrast, nephrin levels were better preserved in ANP(4-23) treated TG mice compared to TG mice treated with PBS vehicle.

Lastly, urinary cGMP excretion tended to be higher in ANP(4-23) treated mice (6.7 ± 1.0 ng/mg creatinine) compared to mice treated with vehicle (4.9 ± 1.0 ng/mg creatinine), but this difference was not statistically significant.

Taken together, these data suggest that: 1. Pharmacologic blockade NPRC may be a useful strategy for treating proteinuric kidney diseases, and 2. Treatment outcomes might be improved by optimizing blockade of the NPRC to more effectively inhibit clearance of NPs from the circulation.

Based on our approved statement of work, these studies are significantly ahead of the estimated target date at the completion of the first year of the grant.

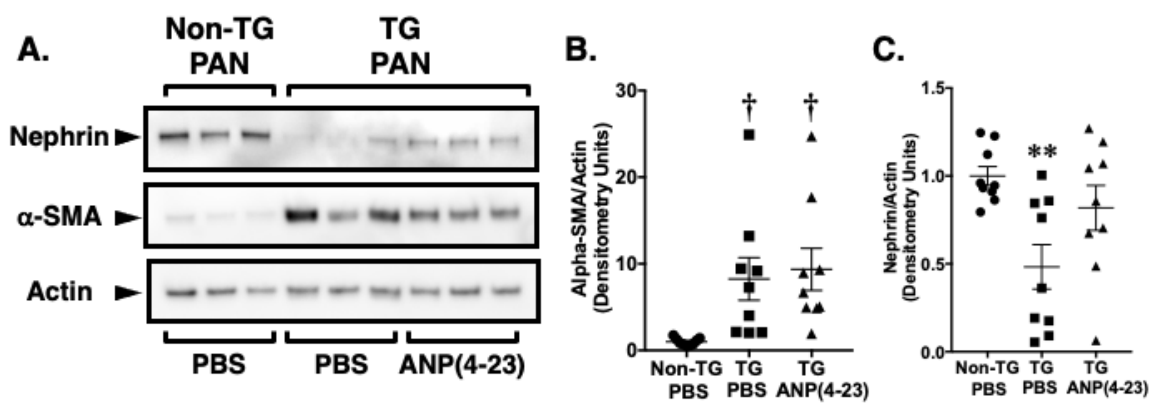

Figure 2. (A &B) Protein levels of α-SMA were significantly increased in both groups of TG mice compare to non-TG controls. (A&C) Nephrin expression was significantly decreased in TG treated with PBS vehicle compared to non-TG mice. In contrast, nephrin expression was netter preserved TG mice treated with ANP(4-23) compared to non-TG controls. †P<0.005 or **P<0.01 versus non-TG controls. Immunoblotting data analyzed by ANOVA followed by Sidak's multiple comparisons test.

Specific aim #2: These studies required specialized equipment available at Duke University including facilities for measuring energy metabolism (indirect calorimetry) and body composition (magnetic resonance imaging) in rodents. Unfortunately, the experiments were impacted by two unanticipated problems. First, a massive gas explosion in Durham, NC, damaged the building housing this specialized equipment. (https://en.wikipedia.org/wiki/2019_Durham_gas_explosion). The building was unstable, and the equipment had to be relocated to another research building, which was accomplished in late 2019. Specialized mice (and controls) were ordered from Jackson Laboratories for the studies in early 2020 and arrived at Duke the end of March, 2020. After arrival, Duke University suspended most animal research due to the COVID-19 pandemic. We were allowed to maintain the line in the Duke animal facility but not to perform the planned experiments. When it was clear that the suspension of routine research activities would not resume for several months, the mice were euthanized because they would not be of the appropriate age for the experiments. Unfortunately, this was extremely costly (>$4000.00) and has significantly delayed completion of these studies.

Based on our approved statement of work, these studies have been delayed significantly and will not be initiated until we are confident that the experiments will not be interrupted by the pandemic.

Specific aim #3: These studies required backcrossing one of our lines onto the appropriate background (FVB/NJ) for the experiments. We are completing the backcrosses and plan to expand the colony over the next 6 months to begin the studies.

Based on our approved statement of work, these experiments are on-schedule at the completion of the first year of the grant.

Opportunities for training and professional development: not applicable

Dissemination of results: Our findings in specific aim #1 were submitted to the American Society of Nephrology National Meeting in 2020.

Plans for next reporting period:

The plans for the next reporting period can be summarized as follows:

1. Complete analysis of the data for specific aim #1. We anticipate that these data will be part of a manuscript that combines specific aim #1 and #3.

2. Initiate the studies in specific aim #2

3. Expand the colony for specific aim #3 and initiate the experiments.

13. Impact

Impact: Blocking NP clearance by NPRC may have a beneficial impact in a wide range of human diseases including obesity, FSGS and the complications of diabetes, such as DKD. Thus, the proposed studies might stimulate the development of NPRC antagonists to treat a broad spectrum of common medical problems.

14. Changes/Problems

Changes in approach and reasons for change:

There are no plans to change the approach of the experiments.

Actual or anticipated problems or delays and actions or plans to resolve them:

As discussed above, initiation of specific aim #2 has been delayed due to a gas explosion that required relocation of the equipment required for the proposed experiments and suspension of routine research activities at Duke University due to the COVID-19 pandemic.

Changes that had a significant impact on expenditures:

As discussed above, routine research activities were suspending at Duke University at the end of March, 2020, due to the COVID-19 pandemic. As a result, we euthanized our Duke colony of specialized mice required for the experiments in specific aim #2 (see above). These mice were purchased from Jackson Laboratories and were extremely costly (>$4000.00).

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

not applicable

Significant changes in use or care of human subjects:

not applicable

15. Products: nothing to report

16. Participants & Other Collaborating Organizations: not applicable

17. Special Reporting Requirements: not applicable

18. Appendices: We have included an abstract submitted to the American Society of Nephrology National Meeting in 2020.

19.

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TITLE: Pharmacologic blockade of the natriuretic peptide clearance receptor ameliorates glomerular disease in an animal model of FSGS

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ABSTRACT BODY:

Background: Glomerular podocytes play a key role in glomerular disease processes. Accumulating evidence suggests that cGMP signaling has podocyte protective effects in kidney diseases (J Am Soc Nephrol 28: 260, 2017). cGMP is produced by nitric oxide and by natriuretic peptides (NPs). NPs are the predominant source of cGMP generation in podocytes. NPs stimulate cGMP production by binding to NP receptors (NPRs). NPRA and NPRB stimulate cGMP generation. In contrast, NPRC binds and degrades NPs. Podocytes express all three NPRs (NPRA, NPRB, and NPRC). We hypothesized that blockade of NPRC would enhance local NP levels, promote cGMP signaling in podocytes and attenuate glomerular injury.

Methods: We blocked clearance of NPs by NPRC using the pharmacologic agent ANP (4-23), which specifically binds NPRC without binding NPRA or NPRB. For the experiments, we used a mouse transgenic (TG) model of focal segmental glomerulosclerosis (FSGS) created in our laboratory (J Clin Invest 125:1913, 2015). These TG mice express a constitutively active Gq α-subunit specifically in podocytes. In these animals, treatment with a single dose of the podocyte toxin puromycin aminonucleoside (PAN) causes robust albuminuria in TG mice, but only mild disease in non-TG animals.

Results: PAN induced heavy proteinuria in vehicle-treated TG mice at day 14 (1426 ± 425 [day 14] vs. 49 ± 19 [baseline] ug/mg creatinine; P = 0.0002). The increase in albuminuria at day 14 was significantly reduced by treatment with ANP(4-23) (1426 ± 425 [vehicle] vs. 383 ± 157 [ANP(4-23)] ug/mg creatinine; P = 0.003).
Treatment with ANP(4-23) also tended to reduce the number of mice with glomerular injury (83% [vehicle] vs 54% [ANP(4-23)]; P = NS). Systolic BP was similar in mice receiving ANP(4-23) and in the vehicle treated group (129 ± 3 [vehicle] vs. 127 ± 3 [ANP(4-23)] mm Hg; P = NS). Urinary cGMP excretion tended to be higher in ANP(4-23) treated mice (6.7 ± 1.0 ng/mg creatinine) compared to mice treated with vehicle (4.9 ± 1.0 ng/mg creatinine), but this difference was not statistically significant.

Conclusion: These data suggest that: 1. Pharmacologic blockade NPRC may be a useful strategy for treating proteinuric kidney diseases, and 2. Treatment outcomes might be improved by optimizing blockade of the NPRC to more effectively inhibit clearance of NPs from the circulation.

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