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Dysregulated NO/PDE5 signaling in the sickle cell mouse lower urinary tract: Reversal by oral nitrate therapy

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ABSTRACT

Aims: Nitric oxide (NO) has a critical, but not well understood, influence in the physiology of the lower urinary tract. We evaluated the effect of NO/phosphodiesterase (PDE)5 signaling in voiding dysfunction in the sickle cell disease (SCD) mouse, characterized by low NO bioavailability.

Main methods: Adult SCD (Sickle) and wild-type (WT) male mice were treated daily with sodium nitrate (10 mM) or vehicle. After 18 days, blood was obtained for nitrite measurement, urethra was collected for organ bath study, and bladder and urethra were collected for Western blot analysis of PDE5 phosphorylation (Ser-92) (activated form). Non-anesthetized mice underwent evaluation of urine volume by void spot assay. eNOS phosphorylation (Ser-1177) and nNOS phosphorylation (Ser-1412) (positive regulatory sites) were evaluated in the bladder and urethra of untreated mice.

Key findings: Sickle mice exhibited decreased eNOS, nNOS, and PDE5 phosphorylation in the bladder and urethra, decreased plasma nitrite levels, increased relaxation of phenylephrine-contracted urethral tissue to an NO donor sodium nitroprusside, and increased total urine volume, compared with WT mice. Nitrate treatment normalized plasma nitrite levels, relaxation of urethra to sodium nitroprusside, PDE5 phosphorylation in the urethra and bladder, and urine volume in Sickle mice.

Significance: Derangement in PDE5 activity associated with basally low NO bioavailability in the bladder and urethra contributes to the molecular basis for voiding abnormalities in Sickle mice. Inorganic nitrate supplementation normalized voiding in Sickle mice through mechanisms likely involving upregulation of PDE5 activity. These findings suggest that interventions targeting dysregulatory NO/PDE5 signaling may ameliorate overactive bladder in SCD.

1. Introduction

Micturition depends on the coordination between the urinary bladder and the urethra. Storage of urine in the bladder requires bladder relaxation during the filling phase, while bladder emptying requires coordinated contraction of the bladder and relaxation of the urethra [1]. Increased contractions of bladder detrusor muscle during the storage phase result in a hypercontractile voiding disorder, the most common cause of overactive bladder syndrome (OAB) [2]. OAB is clinically characterized by urinary urgency with or without urinary urge incontinence and is usually accompanied by urinary frequency and nocturia. The estimated prevalence of OAB is between 11.8 and 24.7% with similar rates in men and women [3].

Detrusor overactivity may result from impairment in detrusor smooth muscle tone, neuronal input, and/or sensory signals originating in the bladder [2]. A wide range of neurotransmitters control urine storage and voiding, including nitric oxide (NO). NO mediates relaxation of urethral and bladder neck smooth muscle [4–7]. NO also contributes to regulation of detrusor tone. Systemic NO synthase (NOS) inhibition, intravesical NO scavenging, and knockout of neuronal NOS (nNOS) and cGMP-dependent protein kinase result in bladder overactivity in rats and mice [8–12], while intravesically or systemically supplied NO donors reduce bladder contraction frequency [13,14]. However, NO donors and cGMP analogues have also been shown to evoke a complex response (relaxant, contractile, or biphasic in isolated human detrusor muscle) [15] or have only a modest relaxing effect on isolated rat and mouse detrusor muscle [16–18]. Furthermore, both NO-mediated cGMP-dependent relaxation [19] and cGMP-independent

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contraction of the detrusor [20,21] have been reported, further complicating the understanding of NO's role in bladder physiology and pathophysiology.

OAB is common in the sickle cell disease (SCD) population, with rates that exceed twice that of age-matched control subjects [22]. Symptoms of OAB in SCD include urinary frequency, urgency and nocturia [22,23]. SCD is caused by a single point mutation in the β-globin gene of hemoglobin leading to polymerization of hemoglobin under hypoxic conditions, red blood cell fragility, hemolysis, vaso-occlusive crises, and inflammation [24]. SCD is also characterized by a chronic state of reduced NO bioavailability [25]. We have shown that homozygous SCD mice, a humanized mouse model of SCD, have decreased endothelial NO bioavailability in the penis associated with decreased phosphodiesterase (PDE)5 function, such that cGMP accumulation occurs upon neurostimulation, which results in excessive cavernosal vasorelaxation and priapism [26–28]. We have also demonstrated that SCD mice exhibit enhanced voiding and non-voiding bladder contractions and produce greater volumes of urine [29]. These basic science findings correlate with clinical observations that both priapism and OAB are common disorders among the SCD population [22].

We hypothesized that chronically aberrant NO regulatory signaling in the bladder and urethra contributes to voiding dysfunction in SCD. We evaluated the effects of NO deficiency in lower urinary tract function using the SCD mouse at baseline and after long-term dietary inorganic nitrate supplementation.

2. Materials and methods

2.1. Animals

Berkeley transgenic sickle cell mice (Sickle) and wild type (WT) male mice, 6–8 months old, were used. Berkeley Sickle mice have deletions of murine α and β globins and a transgene containing human α and β globins [30]. WT C57BL/6 mice were chosen as controls because this represents the predominant background strain for Sickle mice. In some experiments, hemizygous littermate mice were used as an additional control. WT mice and breeding pairs for Sickle mice (strain number 3342) were obtained from Jackson Laboratory (Bar Harbor, ME). Sickle mice were housed and bred in-house. Genotyping was performed by Transnetyx, Inc (Cordova, TN). The SCD phenotype was also confirmed after euthanasia by observation of extremely enlarged spleens [30]. Mice were pathogen free and received routine NIH rodent chow and water. All animal procedures were conducted in accordance with the ethical standards of the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Laboratory Animals, and they complied with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were done under the animal protocol number MO17M06. Mice were anesthetized with 50 mg/kg Ketamine +5 mg/kg Xylazine by intraperitoneal injection. All efforts were made to minimize animal suffering.

2.2. Sodium nitrate supplementation

Sodium nitrate or vehicle sodium chloride (Sigma Aldrich, St. Louis, MO, USA) were added to the drinking water at a concentration of 0.85 g/L and 0.5844 g/L, respectively (about 4.25 mg nitrate/day according to the measured daily drinking water consumption). Sickle and WT mice were treated for 18 days [31]. Water consumption was recorded every second day. At the end of the treatment, blood was obtained by cardiac puncture and tissues were collected for organ bath study (urethra) or Western blot analyses (bladder and urethra) before anesthetized mice (n = 3–7 per group) were sacrificed. Non-anesthetized mice (n = 3) were used for evaluation of urine volume by void spot assay.

2.3. Measurement of plasma nitrite

Blood was collected into heparinized eppendorf tubes at 4 °C. Blood was centrifuged (8000 × *g*) for 10 min, and the resulting plasma supernatant was stored at −80 °C until assay. Plasma nitrite was measured by tri-iodide based reductive chemiliuminescence as previously described [32].

2.4. Measurement of urine volume

For this study, we chose a noninvasive void spot assay as a micturition assessment tool for urinary function in vivo (33). We recently defined an OAB phenotype in Sickle mice using both this assay and more invasive techniques, showing correlative findings by cystometry and void spot assay [29].

Briefly, for measurement of total urine volume by void spot assay, individual mice from communal housing (3–5 animals per cage) were placed on a customized raised wire platform (model #N10SSRWF, Ancare, Bellmore, NY) within a clean, empty cage lined with Whatman Grade 1 filter paper (Fisher Scientific, Waltham, MA) and were provided access to food and water ad libitum. Experiments were performed in triplicate with each animal observed over a 4-h period and then weighed on consecutive days between 9 a.m. and 2 p.m. Voided volume was then evaluated by blinded analysis of the retrieved filter paper as previously described [29,33].

2.5. Western blot analysis

Bladder and urethra were collected from anesthetized mice, snap-frozen in liquid nitrogen, and stored at −80 °C until processed for Western blot analyses. The urethra was sonicated, and the bladder was homogenized as previously described [34]. Protein concentration was determined using the bicinchoninic acid method. Homogenates (30–80 μg) were resolved on 4–20% Tris gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature in PBS (pH 7.4) containing 0.1% Tween-20 and 5% nonfat dry milk, and then probed overnight at 4 °C in PBS containing 0.1% Tween-20 and 3% nonfat dry milk with the following antibodies: rabbit anti-phospho (P)-PDE5 (Ser-92) (1:450 dilution, Fabgennix, Frisco, TX, catalog number PPD5A-140AP), rabbit anti-P-endothelial NOS (eNOS) (Ser-1177) (1:450 dilution, Cell Signaling Technology, Beverly, MA, USA, catalog number 9571S) or rabbit anti-P-nNOS (Ser-1412) (1:7000 dilution, kindly provided by Dr. Solomon Snyder, Johns Hopkins University, Baltimore, MD, USA). Signals were standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; monoclonal antibody at 1:5000 dilution, Santa Cruz Biotechnology, catalog number 15734), eNOS (monoclonal anti-eNOS at 1:1000 dilution, BD Transduction, Laboratories, San Diego, CA, USA; catalog number 610296), or nNOS (polyclonal anti-nNOS at 1:9000 dilution, kindly provided by Dr. Solomon Snyder), on stripped membranes [28,34–36]. Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibodies (1:7000 dilutions, GE Healthcare, catalog numbers NA931V and NA934V) and quantified using NIH Image 1.29 software (National Institutes of Health). Results were expressed relative to those of WT mice treated with vehicle.

2.6. In vitro functional studies and concentration-response curves in urethral smooth muscle strips

Bladder, urethra and prostate were dissected free *en bloc* and immersed in a petri-dish containing Krebs-Henseleit solution. The urethra was isolated by dissection of surrounding soft tissues, and then separated from the bladder. The dissected urethral strips were mounted in a muscle strip myograph system (Danish Myo Technology A/S, Aarhus, Denmark). Organ baths containing 5 ml of Krebs solution were continuously gassed with 95% O_2 and 5% CO_2 at 37 °C. Tissues were then

allowed to equilibrate for 60 min under a resting tension of 2 mN, and the Krebs solution was changed every 15 min. Isometric force was recorded using a PowerLab data acquisition system (Software LabChart, AD Instrument).

Cumulative concentration-response curves were constructed for the NO-donor sodium nitroprusside (SNP, 10 nM - 100 μM) in urethral smooth muscle strips pre-contracted with phenylephrine (10 μM) [37].

2.7. Statistical analysis

GraphPad Prism (Prism v.5, GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. For comparison of Western blot data between treatment groups, one-way analysis of variance, followed by Newman-Keuls multiple comparisons test, was used. For comparison of Western blot data between WT and each treatment group, modified *t*-test was used to compare results in the experimental groups with the normalized control ratio. Data were expressed as the mean ± standard error of the mean (SEM). For comparison of plasma nitrite and urine volume results between groups, a non-parametric Kruskal-Wallis test was used, due to a small number of samples.

To determine the pEC50, nonlinear regression analysis was carried out using GraphPad Prism with the constraint that F = 0. All concentration-response data was fitted to a logistic function in the form: $E = E_{max}/([1 + (10c/10x)n] + F)$, where E is the maximum response produced by agonists; c is the logarithm of the EC_{50}, the concentration of drug that produces a half-maximal response; x is the logarithm of the concentration of the drug; the exponential term, n, is a curve fitting parameter that defines the slope of the concentration–response line, and F is the response observed in the absence of added drug. Relaxation responses were calculated as percentages of the maximal changes from the steady-state contraction produced by phenylephrine. Data are shown as the percentage of relaxation of n experiments, expressed as the mean values ± SEM. In each case, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Protein expressions of P-eNOS (Ser-1177) and P-nNOS (Ser-1412) were decreased in the sickle mouse urethra and bladder

Activated (phosphorylated) forms of eNOS (Ser-1177) and nNOS (Ser-1412) were significantly decreased in the Sickle mouse urethra compared to that of WT mice, and did not differ from that of hemizygous mice (Fig. 1A and B). HEK293 cells transfected with eNOS and stimulated with insulin-like growth factor-1 [34], and major pelvic ganglia (MPG), were used as controls for P-eNOS (Ser-1177) and nNOS, respectively.

Activated (phosphorylated) eNOS (Ser-1177) was significantly decreased in the Sickle and hemizygous mouse bladder compared to that of WT mice (Fig. 2A), while activated (phosphorylated) nNOS was significantly decreased in the Sickle mouse bladder compared to that of WT, and did not differ from that of hemizygous mice (Fig. 2B).

Because hemizygous mice exhibited a partially defective NOS pathway, having NOS changes measured between those of WT and Sickle mice, for the remaining of experiments we used only WT mice as controls.

3.2. Plasma nitrite at baseline and after long-term nitrate administration to sickle and WT mice

Volumes of sodium nitrate and vehicle consumed by mice were not different (6.73 ± 0.49 ml of sodium nitrate/mouse/day vs 6.04 ± 0.29 ml of sodium chloride/mouse/day). Total volumes consumed by WT (5.86 ± 0.45 ml/mouse/day) and Sickle (6.31 ± 0.58 ml/mouse/day) mice also were not different. Body weights of WT and sickle mice were not affected by nitrate treatment: WT + vehicle

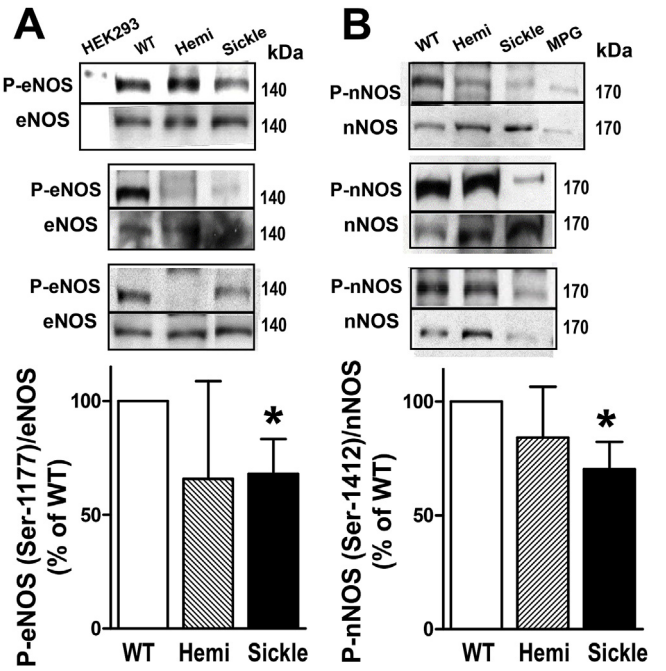

Fig. 1. eNOS phosphorylation on Ser-1177 (A) and nNOS phosphorylation on Ser-1412 (B) were decreased in the urethra of Sickle compared to WT mice. Upper panels are representative Western immunoblots, shown in triplicates, of P-eNOS (Ser-1177), eNOS, P-nNOS (Ser-1412), and nNOS in the urethra of WT, hemizygous, and Sickle mice. Lower panel represents quantitative analysis of P-eNOS/eNOS and P-nNOS/nNOS in the same treatment groups. n = 5–6. *, P < 0.05 vs WT. HEK293 = positive control for P-eNOS (Ser-1177) [34]; MPG = major pelvic ganglia (positive control for nNOS).

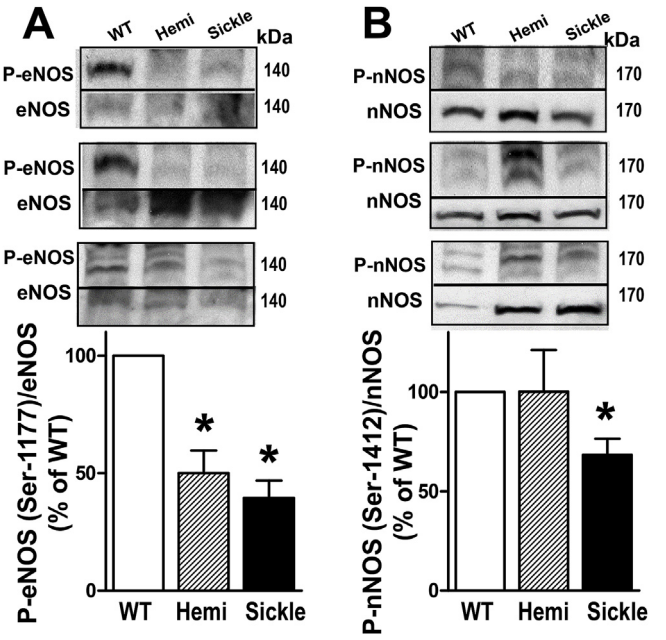

Fig. 2. eNOS phosphorylation on Ser-1177 (A) and nNOS phosphorylation on Ser-1412 (B) were decreased in the bladder of Sickle compared to WT mice. Upper panels are representative Western immunoblots, shown in triplicates, of P-eNOS (Ser-1177), eNOS, P-nNOS (Ser-1412), and nNOS in the bladder of WT, hemizygous, and Sickle mice. Lower panel represents quantitative analysis of P-eNOS/eNOS and P-nNOS/nNOS in the same treatment groups. n = 5. *, P < 0.05 vs WT.

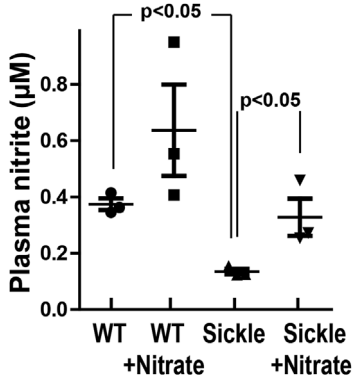

Fig. 3. Plasma nitrite at baseline and after long-term nitrate administration to Sickle and WT mice. Long-term (18 days) treatment of Sickle mice with nitrate (10 mM in drinking water) increased downregulated plasma levels of nitrites. n = 3.

(29.7 ± 0.2 g), WT + nitrate (29.6 ± 0.8 g), Sickle + vehicle (29.5 ± 2.7 g), Sickle + nitrate (27.5 ± 0.9 g). Baseline plasma nitrite levels were significantly lower in Sickle mice compared with that of WT mice (Fig. 3). Long-term nitrate administration increased plasma nitrite levels in Sickle mice to levels found in vehicle-treated WT mice. Nitrate administration also increased plasma nitrite in WT mice; however, this increase was not significant (p = 0.0915).

3.3. Total urine volume was increased in sickle mice, and normalized by long-term nitrate administration

Sickle mice produced significantly greater total volume of urine compared with WT mice by approximately 2-fold (Fig. 4). Long-term nitrate administration significantly decreased total urine volume in Sickle mice to levels found in vehicle-treated WT mice, but did not affect urine volume in WT mice.

3.4. Relaxation of Sickle mouse urethra to an NO donor was increased, and normalized by long-term nitrate administration

The addition of phenylephrine (10 μM) caused submaximal contractions (mN/mg of tissue) of urethral smooth muscle strips that did not significantly differ between groups: WT + vehicle (3.5 ± 0.5), WT + nitrate (1.9 ± 0.8), Sickle + vehicle (2.5 ± 0.5), Sickle + nitrate (3.5 ± 0.4).

The cumulative addition of SNP (10 nM - 100 μM) to phenylephrine-contracted urethral smooth muscle strips produced concentration-dependent relaxations in all mouse groups (Fig. 5A). The maximal response (E_{max}) induced by SNP was significantly higher in urethras of

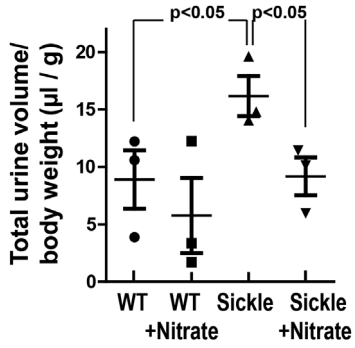

Fig. 4. Total urine volume was increased in Sickle mice and normalized by long-term nitrate administration. Long-term (18 days) treatment of Sickle mice with nitrate (10 mM in drinking water) decreased elevated total urine volume. n = 3.

Sickle compared with WT mice, and was significantly reduced by long-term treatment with nitrate (Fig. 5B). Similarly, SNP pEC_{50} (potency) was significantly higher in urethras of Sickle compared with WT mice (Fig. 5C); long-term treatment with nitrate restored, although not significantly (p = 0.1121), the SNP pEC_{50} in Sickle mice treated with nitrate. No significant changes after nitrate treatment were observed in SNP E_{max} or pEC_{50} in urethras of WT mice.

3.5. Protein expression of P-PDE5 (Ser-92) was decreased in the sickle mouse urethra and bladder, and normalized by long-term nitrate administration

Activated (phosphorylated) PDE5 (Ser-92) was significantly decreased in the Sickle mouse urethra (Fig. 6A) and bladder (Fig. 6B) compared to that of WT mice. Long-term nitrate administration significantly increased phosphorylated PDE5 in the Sickle mouse urethra and bladder to the levels found in vehicle-treated WT mice. Nitrate administration did not affect phosphorylated PDE5 in the WT mouse urethra or bladder.

4. Discussion

In this study, we demonstrate an association between PDE5 dysregulation and basally low NO bioavailability in the bladder and urethra, and impaired lower urinary tract function, in a mouse model of SCD. Long-term oral treatment with inorganic nitrate reversed aberrant signaling of the NO/PDE5 axis and normalized the amount of voided volume, a surrogate for urinary function, in Sickle mice. These results fit with our previous findings that low NO/PDE5 bioavailability in the Sickle mouse penis results in priapism [27,28], and long-term treatment with an NO donor and NO-releasing compound attenuates derangements in erectile function signaling in the penis [38,39]. Overall low NO bioavailability in Sickle mice is supported by our finding that these mice manifest low plasma nitrite levels.

OAB has been clinically observed in the pediatric and adult SCD population [22], but no mechanistic basis has been explained. This study provides evidence that the physiologic sources of NO release, which regulate urethral and detrusor smooth muscle tone and contribute toward normal storage and voiding functions, are defective in the Sickle mouse bladder and urethra. Low NO function in the lower urinary tract conceivably contributes to the molecular basis of abnormal voiding suggestive of detrusor overactivity, as shown here, and fits with an OAB phenotype in Sickle mice [29], as well as other animal models of OAB [8,9,16]. The molecular mechanism underlying the reduction in NOS activity involves decreased phosphorylation of both constitutive NOS isoforms on positive regulatory sites (Ser-1177 and Ser-1412 on eNOS and nNOS, respectively). Whereas prior reports presented conflicting results of increased and decreased protein expressions of total eNOS and nNOS in OAB of rats [40–42], we show decreased activated, phosphorylated eNOS (Ser-1177) and nNOS (Ser-1412) expressions in the Sickle mouse bladder and urethra. Increased protein expressions of total eNOS and nNOS, yet reduced activated/phosphorylated forms of the enzymes, commonly indicate increased expression of a nonfunctional enzyme [28]. eNOS in the urothelium can be activated by phosphorylation on Ser-1177 in response to insulin [43] or mechanical stretch [44] by Akt-dependent mechanisms, resulting in release of NO into the detrusor, cGMP production, and bladder relaxation. The nNOS phosphorylation site 1412 is a target for Akt and protein kinase A in some systems [45], but it is not known what stimuli cause nNOS activation by phosphorylation in the bladder or urethra.

We report down-regulated PDE5 activity in the Sickle mouse bladder and urethra. PDE5, the enzyme that hydrolyzes cGMP and terminates NO action, has been localized in the bladder and urethra to the vascular smooth muscle, striated muscle, and endothelium [46]. Decreased PDE5 activity in the urethra of Sickle mice is also evidenced by heightened urethral relaxation to an NO donor SNP (because the NO

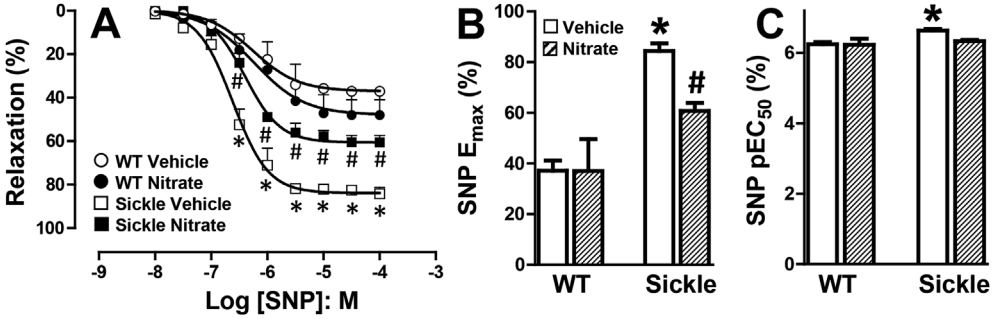

Fig. 5. Relaxation of Sickle mouse urethra to a NO donor was increased, and normalized by long-term nitrate administration. Relaxation response of phenylephrine-contracted urethral strips to SNP was increased in Sickle mice and reversed by long-term nitrate treatment (A). SNP E_{max} (B) and pEC_{50} values (C). n = 3. *, P < 0.05 vs WT + Vehicle; #, P < 0.05 vs Sickle + Vehicle.

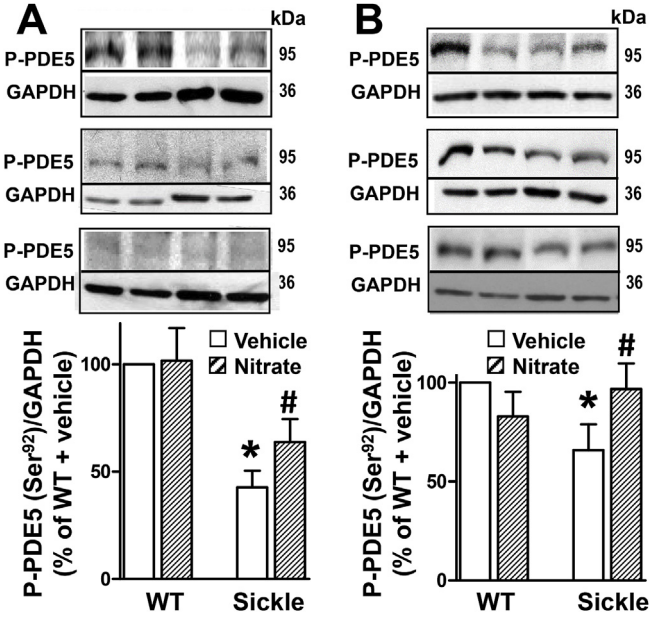

Fig. 6. Protein expression of P-PDE (Ser-92) was decreased in the Sickle mouse urethra and bladder, and normalized by long-term nitrate administration. Long-term (18 days) treatment of Sickle mice with nitrate (10 mM in drinking water) increased downregulated P-PDE5 (Ser-92) expression in the urethra (A) and bladder (B). Upper panels are representative Western immunoblots, shown in triplicates, of P-PDE5 (Ser-92) and GAPDH in the urethra and bladder of WT, WT treated with nitrate, Sickle, and Sickle mice treated with nitrate. Lower panels represent quantitative analyses of P-PDE5 (Ser-92)/GAPDH in the same treatment groups. n = 5–7. *, P < 0.05 vs WT + Vehicle; #, P < 0.05 vs Sickle + Vehicle.

effector, cGMP, cannot be degraded). Low PDE5 activity is conceivably due to low NO bioavailability and absence of the cGMP-dependent feedback mechanism for PDE5 regulation [47,48]. A previous study described reduced protein expression of PDE5 in the bladder of male rats following bladder outlet obstruction-induced bladder overactivity, which has been speculated to be an adaptive response to decreased levels of cGMP; however, no functional relevance was suggested [49].

We next wanted to determine whether targeting dysregulatory NO signaling with inorganic nitrate corrects aberrant NO/PDE5 signaling and normalizes voiding function in Sickle mice. Inorganic nitrate from dietary sources is converted by an NOS-independent enzymatic reduction in vivo to nitrite and then to NO and other bioactive nitrogen oxides. Nitrates' half-life of 6 h allows continuous generation of nitrite [31,50]. In contrast to pharmacological application of the anion nitrite, dietary nitrate is nontoxic even in higher doses. Recent research in animals and humans has shown the beneficial effects of dietary nitrate in promoting cardiovascular health by restoring NO homeostasis in pathological conditions related to a disrupted NO pathway, challenging official recommendations to limit nitrate intake [51]. We found normalized plasma nitrite in Sickle mice and normalized PDE5 activity in the Sickle mouse bladder and urethra after long-term nitrate treatment. The effect of nitrate on PDE5 activity is conceivably due to NO-induced increased accumulation of intracellular cGMP and cGMP-dependent protein kinase G-mediated phosphorylation of PDE5 on Ser-92, which increases PDE5 catalytic activity [48]. Normalized PDE5 activity in the Sickle mouse urethra by nitrate treatment is further evidenced by restored relaxation of phenylephrine-contracted urethral strips to increasing concentrations of the NO donor SNP (because the NO effector cGMP can now be degraded). Normalized NO/PDE5 signaling correlates with restored normal voiding function in Sickle mice, as documented by less excessive total voided volumes. These findings suggest that the recovery of intact NO/cGMP responsiveness and PDE5 regulatory function restores physiologic conditions, a finding which we also observed in the Sickle mouse penis after long-term PDE5 inhibitor and NO donor treatments [27,38,39].

Evidence that NO treatment improved both voiding function (current study) and priapism [38,39] in Sickle mice, two clinical disorders that frequently occur in SCD patients [22], suggests that NO dysregulation and uncontrolled NO/cGMP responsiveness in the penis, bladder, and urethra may define a global pathomechanism for lower genitourinary tract dysfunction. Our findings support the potential that normalizing deranged molecular NO signaling may have therapeutic potential to correct both voiding dysfunction and priapism in SCD.

Although we focused on the effect of NO on PDE5 function in this study, other NO-related effects may also be involved in urinary abnormalities, such as oxidative stress or vasoconstriction. Systemic oxidative stress is increased in SCD patients and Sickle mice [52], and elevated levels of reactive oxygen species have been proposed to contribute to OAB in animal models of obesity, deregulated S-nitrosylation, bladder outlet obstruction, and obstructive sleep apnea [37,53–55]. The RhoA/ROCK contractile pathway also plays an important role in the regulation of urinary bladder smooth muscle contraction and tone. Augmented RhoA/ROCK signaling may result in OAB, as shown in animal models of deregulated S-nitrosylation, bladder outlet obstruction, and hypertension [53,56–58].

Hemizygous mice exhibited partial defects in activated eNOS in the bladder similar to Sickle mice, although, in contrast to Sickle mice, they did not exhibit defects in activated nNOS in the bladder or activated constitutive NOSs in the urethra. We and others previously reported that pathologic abnormalities in hemizygous mice are intermediate between those of control and Sickle mice. For example, hemizygous mice exhibit some of the defects in the testicular steroidogenic pathway and increased oxidative stress in the testis [59] and cremaster muscle [60] similar to Sickle mice. Similar to hemizygous mice, humans with sickle cell trait (carrying one copy of HbS), while usually healthy, may manifest some complications of SCD such as increased frequency of urinary tract infections, kidney disease, pain crises, and leg ulcers [61–63].

The unique feature of our study includes the use of an animal model of natural lower urinary tract dysfunction with chronically low bioavailable NO. This differs from many other animal models of this pathology that result from surgical modification (i.e. urinary outlet

modification), intravesical administration of a chemical or toxin, or induction of neurologic disease [64].

There are several limitations in our study. First, although we documented larger total voided volume amounts in Sickle mice (which we previously correlated with OAB) [29], and its normalization by nitrate treatment, cystometric studies or in vitro functional studies in the bladder would further characterize and corroborate a scientific relationship between NO/PDE5 signaling and the physiologic response. Second, our conclusions may not immediately represent voiding dysfunction in female subjects with SCD, and it is recognized that women with SCD have a greater rate of having OAB symptomatology than men with SCD [22]; further study is necessary to elucidate whether more pronounced disturbances in NO signaling occur in the bladder and urethra of female Sickle mice. Third, we acknowledge the possibility that other abnormalities which are unconnected with NO signaling could also contribute to OAB in transgenic Sickle mice, such as hemolysis-related blood flow disturbances, defects in urine-concentrating ability of the kidney, and metabolic disturbances.

5. Conclusion

Derangements in the PDE5 regulatory pathway and basally low NO bioavailability in the bladder and urethra are molecular conditions associated with voiding dysfunction in Sickle mice. Inorganic nitrate supplementation normalized voiding function in Sickle mice through mechanisms likely involving the upregulation of PDE5 activity in the bladder and urethra. These findings suggest that interventions targeting dysregulatory NO/PDE5 signaling may ameliorate OAB in SCD.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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