JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

NO-releasing nanoparticles ameliorate detrusor overactivity in transgenic sickle cell mice via restored NO/ROCK signaling

Serkan Karakus, Biljana Musicki, Mahantesh S Navati, Joel M. Friedman, Kelvin P. Davies, Arthur L. Burnett

The James Buchanan Brady Urological Institute and Department of Urology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA (S.K., B.M., A.L.B.); Department of Physiology and Biophysics, Albert Einstein College of Medicine, New York, NY, USA (M.S.N., J.M.F., K.P.D.); Department of Urology, Albert Einstein College of Medicine, New York, NY, USA (K.P.D.)

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

a) **The running title:** NO-np ameliorate detrusor overactivity in Sickle Cell Mice
b) **Corresponding Author:** Serkan Karakus, M.D., The James Buchanan Brady Urological Institute, Johns Hopkins School of Medicine, 600 North Wolfe Street, Marburg 414, Baltimore, Maryland 21287-2101, Phone: 410-955-0352, Fax: 410-614-3695, e-mail: skaraku2@jhmi.edu
The number of text pages: 10
The number of tables: 0
The number of figures: 4
The number of references: 46
The number of words in the Abstract: 247
The number of words in the Introduction: 501
The number of words in the Discussion: 990
c) **Abbreviations:** Akt, protein kinase B; cGMP, cyclic guanosine monophosphate; DSM, detrusor smooth muscle; dNOS−/−, combined eNOS and nNOS gene-deficient; eNOS, endothelial nitric oxide synthases; LUT, lower urinary tract; MYPT1, myosin phosphatase target subunit 1; nNOS, neuronal nitric oxide synthase; NO, neuronal nitric oxide; NO-np, NO-releasing nanoparticles; NVCs, non-voiding contractions; NOS, nitric oxide synthase; OAB, overactive bladder; PDE5, phosphodiesterase type 5; PBS, Phosphate-buffered saline; PEG, polyethylene glycol; RhoA, ras homolog gene family, member A; ROCK, rho-associated kinases; SCD, sickle cell disease; VCs, voiding contractions; WT, wild-type.
d) Other

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Sickle cell disease (SCD) is associated with overactive bladder (OAB). Detrusor overactivity, a component of OAB, is present in a SCD mouse, but the molecular mechanisms for this condition are not well defined. We hypothesize that NO/RhoA/ROCK dysregulation is a mechanism for detrusor overactivity and that NO-releasing nanoparticles (NO-np), a novel NO delivery system, may serve to treat this condition. Male adult SCD transgenic, combined eNOS/nNOS knockout (dNOS$^{-/-}$), and wild-type (WT) mice were used. Empty-np or NO-np was injected into the bladder, followed by cystometric studies. The expression levels of phosphorylated eNOS (Ser-1177), Akt (Ser-473), nNOS (Ser-1412), and MYPT1 (Thr-696) were assessed in the bladder. SCD and dNOS$^{-/-}$ mice had a greater ($P<0.05$) number of voiding and non-voiding contractions compared to WT mice, and they were normalized by NO-np treatment. eNOS (Ser-1177) and AKT (Ser-473) phosphorylation were decreased ($P<0.05$) in the bladder of SCD compared to WT mice and reversed by NO-np. Phosphorylation of myosin phosphatase target subunit 1 (P-MYPT1), a marker of the RhoA/ROCK pathway, was increased ($P<0.05$) in the bladder of SCD mice compared to WT and reversed by NO-np. nNOS phosphorylation on positive (Ser-1412) regulatory site was decreased ($P<0.05$) in the bladder of SCD mice compared to WT and was not affected by NO-np. NO-np did not affect any of the measured parameters in WT mice. In conclusion, dysregulation of NO and RhoA/ROCK pathways are associated with detrusor overactivity in SCD mice; NO-np reverses these molecular derangements in the bladder and decreases detrusor overactivity.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Significance Statement

Voiding abnormalities commonly affect SCD patients but are problematic to treat. Clarification of the science for this condition in an animal model of SCD may lead to improved interventions for it. Our findings suggest that novel topical delivery of a vasorelaxant agent nitric oxide into the bladder of these mice corrects overactive bladder by improving deranged bladder physiology regulatory signaling.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

The urinary bladder is a unique organ that has a dual function; it relaxes while filling with urine and contracts to expel urine during micturition. Normal bladder function requires a coordinated and complex interaction between the brain, spinal cord, and lower urinary tract (LUT). Various stimuli induce release of chemical mediators from the urothelium. Disturbances between the nervous system and the urothelial cells may lead to sensory symptoms such as urinary frequency and urgency (Abrams et al., 2017). Nitric oxide (NO) is a urothelial signaling molecule that increases the permeability of urothelium and reduces the activity of detrusor smooth muscle (DSM) via inhibiting the activity of bladder afferent nerves (Lavelle JP et al., 2000; Pandita RK et al., 2000; Parsons CL et al., 2007). However, whether NO exerts direct action on DSM is unclear. Intravesical NO scavenging and systemic nitric oxide synthase (NOS) inhibition result in detrusor overactivity in mice and rats, while NO donors reduce detrusor contraction frequency (Persson K et al., 2000; Monica FZ et al., 2008; Pandita RK et al., 2000; Burnett AL et al., 1997; Kamiyama Y et al., 2008; Ozawa H et al., 1999; Caremel R et al., 2010). On the other hand, spontaneous activity in the DSM may be enhanced by NO through NO/cGMP-independent mechanisms (Meng E et al., 2012; Yanai Y et al., 2008). Furthermore, a complex response (contraction, relaxation, or biphasic) of DSM to NO donors and cGMP analogues has been shown in isolated human and animal detrusor strips (Moon A, 2002). The understanding of NO's role in the physiology and pathophysiology of the LUT remains unclear and warrants further studies.

Detrusor overactivity represents a major underlying pathophysiology of overactive bladder (OAB) syndrome and is defined by urodynamic criteria as involuntary detrusor contractions during the filling phase. The prevalence of OAB has been estimated to be between 16.5–23.2% in the general population of the United States, and as high as 40% in patients with sickle cell disease (SCD) (Anele UA et al., 2016; Portocarrero ML et al., 2012; Powell LC et al., 2018). SCD is an inherited disorder characterized by an abnormal structure of one of the globin

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

chains of the hemoglobin molecules, resulting in multiple clinical presentations. The chronic state of reduced NO bioavailability plays a significant role in the pathogenesis of SCD, and prior studies have implicated dysregulation of the NO signaling pathway in genitourinary dysfunctions in SCD, such as priapism and OAB (Silva FH et al., 2016; Karakus S et al., 2019; Musicki B et al., 2019).

In our previous study, we showed that chronically dysregulated NO signaling in LUT contributes to detrusor overactivity and urethral dysfunction in SCD, while long-term dietary inorganic nitrate supplementation corrected the OAB phenotype by reversing NO deficiency (Musicki B et al., 2019). In this study, we evaluated the effect of NO deficiency and NO replacement with a novel NO therapy, NO-nanoparticles (NO-np), on voiding function and molecular signaling in the bladder using two mouse models of NO deficiency: transgenic SCD mice and combined eNOS and nNOS gene-deficient (dNOS$^{-/-}$) mice.

Material and Methods

Animals. Male 3.3 - 6 months old, age-matched SCD transgenic, dNOS$^{-/-}$, and wild-type (WT) mice were used for the following evaluations: cystometry (3.6-6 month-old); Western blot analysis (3.3-5.6 month-old). Transgenic SCD mouse breeding pairs (strain number 3342) were obtained from Jackson Laboratory and generated by knockout of mouse α and β globins and insertion of a single transgene that expresses human α and β sickle globin (Pászty C et al., 1997). SCD mice were bred in-house. Genotyping was performed by Transnetyx, Inc. (Cordova, TN, USA). Adult male dNOS$^{-/-}$ mice, originally obtained from Dr. Paul L. Huang and bred in-house were utilized as a secondary and confirmatory model of NO deficiency (Huang PL et al. 1993). WT; C57BL/6 male mice (The Jackson Laboratory, Bar Harbor, ME, USA) were utilized as controls representing the strains for the predominant genetic background of the transgenic SCD and dNOS$^{-/-}$ mice. Mice were kept in a germ-free animal facility with a 12/12-hour day/night

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

cycle. All animal studies were conducted in accordance with ethical standards of the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Animals.

Nanoparticles preparation. The synthesis of NO-np and empty-np was recently reported (Friedman AJ et al., 2011). A fine powder comprised of 10 nm empty nanoparticles or 10nM NO nanoparticles was mixed with saline (0.9 mg/ml and 5 mg/ml, respectively) and afterwards with PEG solution (12.5 µl/mg and 10 µl/mg, respectively) just before use (Han G et al., 2010; Tar M et al., 2014). Once exposed to an aqueous environment, the hydrogel properties of the composite allow opening of the water channels inside the particles, facilitating the release of NO, which lasts up to several hours.

Cystometry. Thirty-seven mice (n = 4-9/group) underwent cystometry. Intraperitoneal injection of urethane (1.8 mg/kg) was used for anesthesia. Once the bladder was exposed via lower abdominal laparotomy, mice were allowed to stabilize for 20-30 minutes before NO-np injections. After emptying the bladder, 50 µl of empty-np or NO-np dispersion was injected by a 31-gauge needle into the bladder. A 25-gauge butterfly needle that was connected to a two-way tap was then inserted into the bladder dome through the same needle hole after 10 minutes. One of these ports was connected to a pressure transducer and the other port was connected to an infusion pump. Cystometry was continuously performed for 30 minutes by infusing saline in the bladder at 10 µl per minute. The parameters assessed were peak pressure (maximum pressure observed during a micturition cycle), bladder capacity (maximum volume infused into the emptied bladder before urine expulsion, assessed at the end of the cystometry), compliance (change in volume/change in baseline and threshold pressures before first voiding contraction occurred), and the frequency of voiding (VCs) and non-voiding (NVCs) contractions. VCs were defined as rises in intravesical pressure that exceeded 4 mmHg and were associated with a saline expulsion from the urethra (micturition). The contractions that were not accompanied by

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

micturition were defined as NVCs (Leiria LO et al., 2013; Karakus S et al., 2019). Cystometry data were recorded using the DI-190 system (Dataq Instruments, Akron, OH, USA).

Western blot analysis. Bladder samples were collected 40 minutes after intravesical injection of 50 µl of empty-np or 50 µl of NO-np from a separate group of anesthetized mice (n=24; 4-10 mice/group) which did not undergo cystometric evaluation. The tissue was snap-frozen in liquid nitrogen, and stored at −80° C until processed for Western blot analyses. The bladder was homogenized as previously described (Karakus S et al., 2017). Protein concentration was determined using the bicinchoninic acid method. Homogenates (90 µg) were resolved on 4-20% Tris gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour 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)-endothelial NOS (eNOS) (Ser-1177) (1:450 dilution, Cell Signaling Technology, Beverly, MA, USA, catalog number 9571S), rabbit anti-P-nNOS (Ser-1412) (1:7,000 dilution, kindly provided by Dr. Solomon Snyder, Johns Hopkins University, Baltimore, MD, USA) (Hurt KJ et al., 2012), rabbit anti- P-myosin phosphatase target subunit 1(MYPT1) (Thr-696) (dilution 1:1000, Cell Signaling Technology, Beverly, MA, USA, catalog number 5163), and rabbit anti-P-Akt (Ser-473) (dilution 1:1000, Cell Signaling Technology, Beverly, MA, USA, catalog number 9271). Signals were standardized to eNOS (monoclonal anti-eNOS at 1:1,000 dilution, BD Transduction, Laboratories, San Diego, CA, USA; catalog number 610296), nNOS (polyclonal anti-nNOS at 1:9,000 dilution, kindly provided by Dr. Solomon Snyder), MYPT1 (polyclonal anti-MYPT1 at 1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA, catalog number sc-25618), or Akt (polyclonal anti-Akt at 1:1,000 dilution, Cell Signaling Technology, Beverly, MA, USA, catalog number 9272). Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibodies (1:7,000 dilutions, GE Healthcare, catalog numbers NA931V and NA934V) and

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

quantified using NIH Image 1.29 software (National Institutes of Health). Results were expressed relative to those of WT mice treated with empty-np.

Statistical Analysis. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by post hoc analysis using the Tukey-Kramer test (Prism v.5, GraphPad Software, San Diego, CA USA). For comparison of Western blot data between WT mice treated with empty-np and each treatment group, a modified t test was used to compare the experimental groups with the normalized control ratio. A value of $P<0.05$ was considered statistically significant.

Results

NO-np improved abnormal cystometric voiding and non-voiding frequencies in SCD and dNOS$^{-/-}$ mice. Cystometrogram tracings showed that SCD and dNOS$^{-/-}$ mice had a greater number of VCs and NVCs and low compliance compared to WT mice (Fig. 1). Both voiding (Fig. 2A) and non-voiding (Fig. 2B) frequencies were increased ($p<0.05$) in dNOS$^{-/-}$ and SCD mice compared to that of WT mice. Bladder compliance was decreased ($p<0.05$) in dNOS$^{-/-}$ and SCD mice compared to that of WT mice (Fig. 2C). Peak pressure was decreased ($p<0.05$) in dNOS$^{-/-}$, and slightly decreased in SCD compared to that of WT mice ($p=0.0565$) (Fig. 2D). There were no significant differences in bladder capacity among groups (Fig. 2E). Treatment of SCD and dNOS$^{-/-}$ mice with NO-np decreased ($P < 0.05$) the number of VCs and NVCs to levels measured in WT mice (Figs. 1 and 2). NO-np treatment did not affect bladder compliance, peak pressure, and capacity in either SCD or dNOS$^{-/-}$ mice. NO-np treatment did not affect cystometric parameters in the bladder of WT mice.

Protein expressions of P-eNOS (Ser-1177), P-Akt (Ser-473) and P-nNOS (Ser-1412) were decreased in the SCD mouse bladder; protein expressions of P-eNOS (Ser-1177) and P-Akt

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

(Ser-473) were normalized by NO-np treatment. Activated (phosphorylated) forms of eNOS (Ser-1177) and Akt (Ser-473) were decreased ($P < 0.05$) in the SCD mouse bladder compared to that of WT mice, and were increased ($P < 0.05$) by NO-np treatment to levels similar to that measured in WT mice (Fig. 3A and 3B). Activated, phosphorylated nNOS (Ser-1412) was also decreased ($P < 0.05$) in the SCD mouse bladder compared to that of WT mice, and was not affected by NO-np treatment. (Fig. 3C). NO-np administration did not affect phosphorylated eNOS, nNOS, or Akt in the WT mouse bladder.

Protein expression of P-MYPT1 was increased in the SCD mouse bladder and normalized by NO-np treatment. The ratio of P-MYPT1 (Thr-696) to total MYPT1, reflecting RhoA/Rho kinase (ROCK) activity, was increased ($P<0.05$) in the SCD mouse bladder compared to that of WT mice. NO-np treatment decreased ($P<0.05$) protein expression of P-MYPT1 in the SCD mouse bladder to levels similar to that measured in WT mice (Fig. 3D). NO-np administration did not affect phosphorylated MYPT1 in the WT mouse bladder.

Discussion

This study demonstrates that intravesical application of NO-np, a new NO-delivery system, reversed OAB phenotype in a SCD mouse model of chronic NO deficiency, as documented by decreased voiding and non-voiding contraction frequencies. This effect of NO-np is associated with normalization of molecular signaling in the bladder involving Akt/NOS relaxing and RhoA/ROCK contractile signaling pathways. The lack of NO-np effect on NO signaling in WT animals is in line with previous findings in the penis of healthy adult rats and healthy adult men with other NO stimulating compounds such as PDE5 inhibitors (Musicki B et al., 2005; Mondaini N et al., 2003). Although NO is understood to be a weak relaxing agent in the bladder under physiologic conditions, its effect may actually be more evident under pathologic conditions.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

In this study, we confirmed our recent finding of OAB phenotype in a SCD mouse model (Karakus S et al., 2019; Musicki B et al., 2019), which is in line with clinical observations that pediatric and adult populations of SCD patients exhibit urinary symptoms such as OAB and nocturnal enuresis (Anele UA et al., 2016). We obtained similar cystometric results using another mouse model of chronic NO depletion, the dNOS knockout mouse, further substantiating the importance of NO deficiency in the pathogenesis of OAB. Here, we provide an explanation for the derangement in molecular signaling resulting in OAB, using a humanized SCD animal model: activities of both constitutive NOS isoforms are decreased, while the RhoA/ROCK contractile pathway is increased in the bladder, both of which contribute to enhanced detrusor overactivity.

The molecular mechanism underlying the reduction in NOS activity in the SCD mouse bladder involves decreased phosphorylation of both constitutive NOS isoforms on positive regulatory sites (Ser-1177 and Ser-1412 on eNOS and nNOS, respectively) and decreased phosphorylation of Akt, an activator of eNOS. These results confirm and extend our recent findings (Musicki B et al., 2019).

We show here for the first time that the SCD mouse bladder exhibits increased activity of the RhoA/ROCK contractile pathway. We documented this by demonstrating increased phosphorylation of MYPT1, a surrogate marker of the RhoA/ROCK pathway activity. MYPT1 is a regulatory subunit of myosin light chain phosphatase, which dephosphorylates the myosin light chain and initiates relaxation of smooth muscle cells at low calcium levels (Grassie ME et al., 2011; Gallagher PJ et al., 1997). Phosphorylation of MYPT1 on Thr-696 by ROCK inhibits the phosphatase activity of myosin light chain phosphatase and thereby causes smooth muscle contraction. Components of the RhoA/ROCK signaling pathway are expressed in the detrusor and play important roles in regulating detrusor contraction and tone (Teixeira CE et al., 2007). Previous studies showed that RhoA/ROCK activity was increased in the OAB bladder in animal models of S-nitrosoglutathione reductase (GSNOR) deficiency, bladder outlet obstruction, and

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

hypertension (Akakpo W et al., 2017; Teixeira CE et al., 2007; Wróbel A et al., 2017). An inverse functional relationship exists between the NO/cGMP/PKG and RhoA/ROCK signaling pathways (Laufs U et al., 1998). Activation of RhoA/ROCK decreases both eNOS protein expression and Akt-dependent eNOS phosphorylation on Ser-1177 (Ming XF et al., 2002; Bolz SS et al., 2003). Conversely, cGMP-dependent PKG phosphorylates RhoA on Ser-188 and prevents its translocation to the membrane, thus preventing its activation and inhibiting the RhoA/ROCK pathway (Cicek FA et al., 2013; Kizub IV et al., 2010). While the mechanism of increased RhoA/ROCK activity in OAB in SCD is not known, it is plausible that it is induced by chronic low NO bioavailability. Upregulated activity of the RhoA/ROCK pathway in the SCD mouse bladder conceivably increases smooth muscle contractility and contributes to the OAB phenotype.

We next used a novel NO delivery system, not described before for intravesical NO delivery, to replenish NO bioavailability in the SCD mouse bladder. NO-np is a true NO generator, not an NO-donating compound with potential cytotoxicity, inadequate release capacity, and instability, and allows localized delivery of the gaseous NO. The nanoparticles uniquely facilitate the formation of NO from nitrite salt through a stable and potent NO intermediate, N_2O_3. NO-np are stable and produce no cytotoxicity or widespread adverse effects (Tar M et al., 2014). Their previous use in the penis, namely for treatment of erectile dysfunction associated with aging, diabetes, and cavernous nerve injury (Han G et al., 2010; Draganski A et al., 2018), indicates their effectiveness in NO delivery. We show here that NO-np treatment reversed detrusor overactivity in both SCD and $dNOS^{-/-}$ mouse models and, as expected with only short-term treatment, did not affect bladder capacity, peak pressure, or compliance. This effect of NO-np treatment was associated with a reversal of downregulated Akt/eNOS activities, and reversal of upregulated ROCK activity, in the SCD mouse bladder. We propose that increased local NO by NO-np treatment resulted in enhanced blood flow and shear forces on the urothelium, activated Akt/eNOS (Ser-1177) phosphorylation cascade, and

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

promoted NO release and relaxation of the bladder (Dimmeler S et al., 1999; Sriram K et al., 2016; Hurt KJ et al., 2002). Increased NO may further downregulate the contractile ROCK pathway activity in the bladder, further improving bladder relaxation (Fig. 4).

The nNOS phosphorylation site 1412 is a target for Akt and protein kinase A in some systems (Forstermann U et al., 2012). We found no effect of NO-np on nNOS phosphorylation on Ser-1412, suggesting that the beneficial effects of NO in ameliorating the OAB phenotype in the SCD mouse is apparently not mediated by increased nNOS phosphorylation, at least with short-term NO-np treatment. While several studies reported higher leak point pressure and bladder capacity, and absent urethral relaxation in mice lacking nNOS (Burnett AL et al., 1997; Sutherland RS et al., 1997), the role of nNOS activation by phosphorylation in the LUT awaits further evaluation.

There were several possible limitations of our study. First, SCD and $dNOS^{-/-}$ mice likely possess metabolic or nephrological abnormalities as well as hemolysis related blood flow disturbances that may contribute to voiding dysfunction (Morishita T et al., 2005). Additionally, utilization of the transgene or gene knockout models may have produced effects independent of NO signaling affecting voiding function in these mice. Second, we did not directly quantify NO release into the bladder. A separate study is warranted to elucidate the nanoparticles penetration profiles in the bladder. Third, further studies are also needed to delineate possible differences in bladder morphology between the WT and SCD mice. Fourth, our study was limited to male SCD mice. Possible sex differences in NO deficiency in OAB can be further investigated using female SCD mice.

Our results demonstrate that SCD mice exhibit an OAB phenotype due to NOS dysregulation and upregulation of the RhoA/ROCK contractile pathway in the bladder; NO-np normalized detrusor overactivity while reversing these molecular abnormalities in the bladder. Our findings suggest the topical application of NO-np as a novel approach for treatment of OAB

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

by targeting the NO signaling pathway. While our studies show short term effects of NO-np, the potential long-term beneficial effect on OAB awaits further investigation.

Acknowledgments

None.

Authorship Contributions

Participated in research design: Karakus, Burnett

Conducted experiments: Karakus

Contributed new reagents or analytic tools: Navati, Friedman, Davies

Performed data analysis: Karakus

Wrote or contributed to the writing of the manuscript: Karakus, Musicki, Burnett

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

References

Abrams P, Cardozo L, Wagg A, Wein A. (2017) Incontinence 6th Edition. ICI-ICS. International Continence Society, Bristol UK, ISBN: 978-0956960733.

Akakpo W, Musicki B, Burnett AL (2017) cAMP-dependent regulation of RhoA/Rho-kinase attenuates detrusor overactivity in a novel mouse experimental model. BJU Int 120: 143-151.

Anele UA, Morrison BF, Reid ME, Madden W, Foster S, and Burnett AL (2016) Overactive bladder in adults with sickle cell disease. Neurourol Urodyn 35: 642-646.

Bolz SS, Vogel L, Sollinger D (2003) Nitric oxide-induced decrease in calcium sensitivity of resistance arteries is attributable to activation of the myosin light chain phosphatase and antagonized by the RhoA/Rho kinase pathway. Circulation 107: 3081-7.

Burnett A.L., D.C. Calvin, S.L. Chamness, J.X. Liu, R.J. Nelson, S.L. Klein, V.L. Dawson, T.M. Dawson, S.H. Snyder (1997) Urinary bladder-urethral sphincter dysfunction in mice with targeted disruption of neuronal nitric oxide synthase models idiopathic voiding disorders in humans, Nat. Med. 3: 571-574.

Caremel R., S. Oger-Roussel, D. Behr-Roussel, P. Grise, F.A. Giuliano (2010) Nitric oxide/cyclic guanosine monophosphate signaling mediates an inhibitory action on sensory pathways of the micturition reflex in the rat, Eur. Urol. 58: 616-625.

Cicek FA, Kandilci HB, Turan B (2013) Role of ROCK upregulation in endothelial and smooth muscle vascular functions in diabetic rat aorta. Cardiovasc Diabetol 12: 51.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM (1999)Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 399: 601-5.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Draganski A, Tar MT, Villegas G, Friedman JM, Davies KP (2018) Topically Applied Curcumin-Loaded Nanoparticles Treat Erectile Dysfunction in a Rat Model of Type-2 Diabetes. J Sex Med 15: 645-653.

Forstermann U., W.C. Sessa (2012) Nitric oxide synthases: regulation and function, Eur. Heart. J 33: 829-837.

Friedman AJ, Han G, Navati MS, Chacko M, Gunther L, Alfieri A, Friedman JM (2008) Sustained release nitric oxide releasing nanoparticles: characterization of a novel delivery platform based on nitrite containing hydrogel/glass composites. Nitric Oxide 19: 12-20.

Gallagher PJ, Herring BP, Stull JT (1997) Myosin light chain kinases. J Muscle Res Cell Motil 18: 1–16.

Grassie ME, Moffat LD, Walsh MP, MacDonald JA (2011) The myosin phosphatase targeting protein (MYPT) family: a regulated mechanism for achieving substrate specificity of the catalytic subunit of protein phosphatase type 1δ. Arch Biochem Biophys 510: 147–59.

Han G, Tar M, Kuppam DS, Friedman A, Melman A, Friedman J, Davies KP (2010) Nanoparticles as a novel delivery vehicle for therapeutics targeting erectile dysfunction. J Sex Med 7: 224-33.

Huang PL, Dawson TM, Bredt DS, Snyder SH, and Fishman MC (1993) Targeted disruption of the neuronal nitric oxide synthase gene. Cell 75: 1273-1286.

Hurt KJ, Musicki B, Palese MA, Crone JK, Becker RE, Moriarity JL, Snyder SH, Burnett AL (2002) Akt-dependent phosphorylation of endothelial nitric-oxide synthase mediates penile erection. Proc Natl Acad Sci U S A 99: 4061-6.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Hurt KJ, Sezen SF, Lagoda GF, Musicki B, Rameau GA, Snyder SH, Burnett AL (2012) Cyclic AMP-dependent phosphorylation of neuronal nitric oxide synthase mediates penile erection. Proc Natl Acad Sci U S A 109:16624-9.

Kamiyama Y., S. Muto, H. Masuda, H. Ide, N. Ishizuka, K. Saito, S. Horie (2008) Inhibitory effects of nicorandil, a K ATP channel opener and a nitric oxide donor, on overactive bladder in animal models, BJU. Int. 101: 360-365.

Karakus S, Musicki B, La Favor JD, Burnett AL 2017 cAMP-dependent post-translational modification of neuronal nitric oxide synthase neuroprotects penile erection in rats. BJU Int. 120: 861-872.Karakus S, Anele UA, Silva FH, Musicki B, Burnett AL (2019) Urinary dysfunction in transgenic sickle cell mice: model of idiopathic overactive bladder syndrome. Am J Physiol Renal Physiol 317: 540-546.

Kizub IV, Pavlova OO, Johnson CD (2010) Rho kinase and protein kinase C involvement in vascular smooth muscle myofilament calcium sensitization in arteries from diabetic rats. Br J Pharmacol 159: 1724-31.

Laufs U, Liao JK (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. J Biol Chem 273: 24266-71.

Lavelle JP, Meyers SA, Ruiz WG, Buffington CA, Zeidel ML, and Apodaca G (2000) Urothelial pathophysiological changes in feline interstitial cystitis: a human model. Am J Physiol Renal Physiol 278: 540-553, 2000.

Leiria L.O., C. Sollon, F.R. Báu, Mónica FZ, D'Ancona CL, De Nucci G, Grant AD, Anhê GF, Antunes E (2013) Insulin relaxes bladder via PI3K/AKT/eNOS pathway activation in mucosa: unfolded protein response-dependent insulin resistance as a cause of obesity-associated overactive bladder. J. Physiol 591: 2259-2273.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Meng E, Young JS, Cha TL, Sun GH, Yu DS, and Brading AF (2012) Neuronal-derived nitric oxide modulates the activity of mouse detrusor smooth muscle. Neurourol Urodyn 31: 572-578.

Ming XF, Viswambharan H, Barandier C (2002) Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. Mol Cell Biol 22: 8467-77.

Mónica F.Z., A.A. Bricola, F.R. Báu, L.L. Freitas, S.A. Teixeira, M.N. Muscará, F.M. Abdalla, C.S. Porto, G. De Nucci, A. Zanesco, E. Antunes (2008) Long-term nitric oxide deficiency causes muscarinic supersensitivity and reduces beta(3)-adrenoceptor-mediated relaxation, causing rat detrusor overactivity, Br. J. Pharmacol. 153:1659-1668.

Mondaini N, Ponchietti R, Muir GH, Montorsi F, Di Loro F, Lombardi G, and Rizzo M (2003) Sildenafil does not improve sexual function in men without erectile dysfunction but does reduce the postorgasmic refractory time. Int J Impot Res 15: 225–228.

Moon A (2002) Influence of nitric oxide signaling pathways on pre-contracted human detrusor smooth muscle in vitro. BJU international 89: 942-949.

Morishita T, Tsutsui M, Shimokawa H, Sabanai K, Tasaki H, Suda O, Nakata S, Tanimoto A, Wang KY, Ueta Y, Sasaguri Y, Nakashima Y, Yanagihara N (2005) Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms. Proc Natl Acad Sci U S A 102: 10616-10621.

Musicki B, Champion HC, Becker RE, Liu T, Kramer MF, Burnett AL (2005) Erection capability is potentiated by long-term sildenafil treatment: role of blood flow-induced endothelial nitric-oxide synthase phosphorylation. Mol Pharmacol 68: 226-32.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Musicki B, Anele UA, Campbell JD, Karakus S, Shiva S, Silva FH, Burnett AL (2019) Dysregulated NO/PDE5 signaling in the sickle cell mouse lower urinary tract: Reversal by oral nitrate therapy. Life Sci 238: 116922.

Ozawa H., M.B. Chancellor, S.Y. Jung, T. Yokoyama, M.O. Fraser, Y. Yu, W.C. de Groat, N. Yoshimura (1999) Effect of intravesical nitric oxide therapy on cyclophosphamide-induced cystitis, J. Urol. 162: 2211–2216.

Pandita RK, Mizusawa H, and Andersson KE (2000) Intravesical oxyhemoglobin initiates bladder overactivity in conscious, normal rats. J Urol 164: 545-550.

Parsons CL. The role of the urinary epithelium in the pathogenesis of interstitial cystitis/prostatitis/urethritis (2007) Urology 69: 9-16.

Pászty C, Brion CM, Manci E, Witkowska HE, Stevens ME, Mohandas N, Rubin EM (1997) Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. Science 278:876–878.

Persson K, Pandita RK, Aszodi A, Ahmad M, Pfeifer A, Fassler R, and Andersson KE (2000) Functional characteristics of urinary tract smooth muscles in mice lacking cGMP protein kinase type I. Am J Physiol Regul Integr Comp Physiol 279: R1112-1120.

Portocarrero ML, Portocarrero ML, Sobral MM, Lyra I, Lordelo P, and Barroso U, Jr (2012) Prevalence of enuresis and daytime urinary incontinence in children and adolescents with sickle cell disease. J Urol 187: 1037-1040.

Powell LC, Szabo SM, Walker D, and Gooch K (2018) The economic burden of overactive bladder in the United States: A systematic literature review. Neurourol Urodyn 37: 1241-1249.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Silva FH, Karakus S, Musicki B, Matsui H, Bivalacqua TJ, Dos Santos JL, Costa FF, Burnett AL (2016) Beneficial Effect of the Nitric Oxide Donor Compound 3-(1,3-Dioxoisoindolin-2-yl)Benzyl Nitrate on Dysregulated Phosphodiesterase 5, NADPH Oxidase, and Nitrosative Stress in the Sickle Cell Mouse Penis: Implication for Priapism Treatment. J Pharmacol Exp Ther 359: 230-237.

Sriram K, Laughlin JG, Rangamani P, Tartakovsky DM (2016) Shear-Induced Nitric Oxide Production by Endothelial Cells. Biophys J 111: 208-21.

Sutherland RS, Kogan BA, Piechota HJ, Bredt DS (1997) Vesicourethral function in mice with genetic disruption of neuronal nitric oxide synthase. J Urol 157: 1109-16.

Tar M, Cabrales P, Navati M, Adler B, Nacharaju P, Friedman AJ, Friedman J, Davies KP (2014) Topically applied NO-releasing nanoparticles can increase intracorporal pressure and elicit spontaneous erections in a rat model of radical prostatectomy. J Sex Med. 11: 2903-14.

Teixeira CE, Jin L, Priviero FB, Ying Z, Webb RC (2007) Comparative pharmacological analysis of Rho-kinase inhibitors and identification of molecular components of Ca2+ sensitization in the rat lower urinary tract. Biochem Pharmacol 74: 647-58.

Wróbel A, Doboszewska U, Rechberger E, Rojek K, Serefko A, Poleszak E, Skalicka-Woźniak K, Dudka J, Wlaź P (2017) Rho kinase inhibition ameliorates cyclophosphamide-induced cystitis in rats. Naunyn Schmiedebergs Arch Pharmacol 390: 613-619.

Yanai Y, Hashitani H, Hayase M, Sasaki S, Suzuki H, and Kohri K (2008) Role of nitric oxide/cyclic GMP pathway in regulating spontaneous excitations in detrusor smooth muscle of the guinea-pig bladder. Neurourol Urodyn 27: 446-453.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Grants

The study was supported by grant from the National Institutes of Health, USA [NIH/NIDDK grant R56DK114095 to A.L.B.].

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Representative cystometrogram traces in WT + Empty-np, WT + NO-np, dNOS$^{-/-}$ + Empty-np, dNOS$^{-/-}$ + NO-np, SCD + Empty-np, and SCD + NO-np. dNOS$^{-/-}$ and SCD mice exhibit unstable bladder contractions. Thick arrows indicate non-voiding contractions, thin arrows indicate voiding contractions.

Figure 2. Effect of NO-np on cystometric parameters (voiding frequency [A], non-voiding frequency [B], compliance [C], peak pressure [D], and capacity [E]) in WT, dNOS$^{-/-}$, and SCD mice. Each bar represents the mean ± SEM for 4-9 mice in each group. *P< 0.05 versus WT, #P< 0.05 versus Empty-np.

Figure 3. Upper panels are representative Western immunoblots of P-eNOS (Ser-1177), eNOS, P-Akt (Ser-473), Akt, P-nNOS (Ser-1412), nNOS, P-MYPT1, and MYPT1 in the bladder of WT + Empty-np, WT + NO-np, SCD + Empty-np, and SCD + NO-np. Lower panel represents quantitative analysis of P-eNOS (Ser-1177), eNOS, P-Akt (Ser-473), Akt, P-nNOS (Ser-1412), nNOS, P-MYPT1, and MYPT1 in the same treatment groups. Each bar represents the mean ± SEM. for 4-10 mice in each group. *P<0.05 versus WT, #P<0.05 versus Empty-np.

Figure 4. Proposed mechanism of NO-np action on amelioration of detrusor overactivity in the SCD mouse

The molecular mechanism underlying detrusor overactivity in the SCD mouse involves the reduction in Akt/eNOS activities (reduced phosphorylation of Akt on Ser-473 and eNOS on Ser-1177) and increased RhoA/ROCK contractile pathway (increased phosphorylation of MYPT1, a regulatory subunit of MLCP, on Thr-696). NO-np treatment increases Akt/eNOS activities and decreases RhoA/ROCK activity in the SCD mouse bladder by enhancing blood flow and shear forces on the urothelium. Decreased phosphorylation of MYPT1 activates MLCP, which dephosphorylates the MLC and initiates relaxation of smooth muscles. Normalized Akt/eNOS and RhoA/ROCK activities ameliorate detrusor overactivity.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

Abbreviations: Akt = protein kinase B; eNOS = endothelial nitric oxide synthases; MLC = myosin light chain; MLCK = myosin light chain kinase; MLCP = myosin light chain phosphatase; MYPT1 = myosin phosphatase target subunit 1; NO-np = NO-releasing nanoparticles; P = phosphorylation; RhoA = ras homolog gene family, member A; ROCK = rho-associated kinase.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

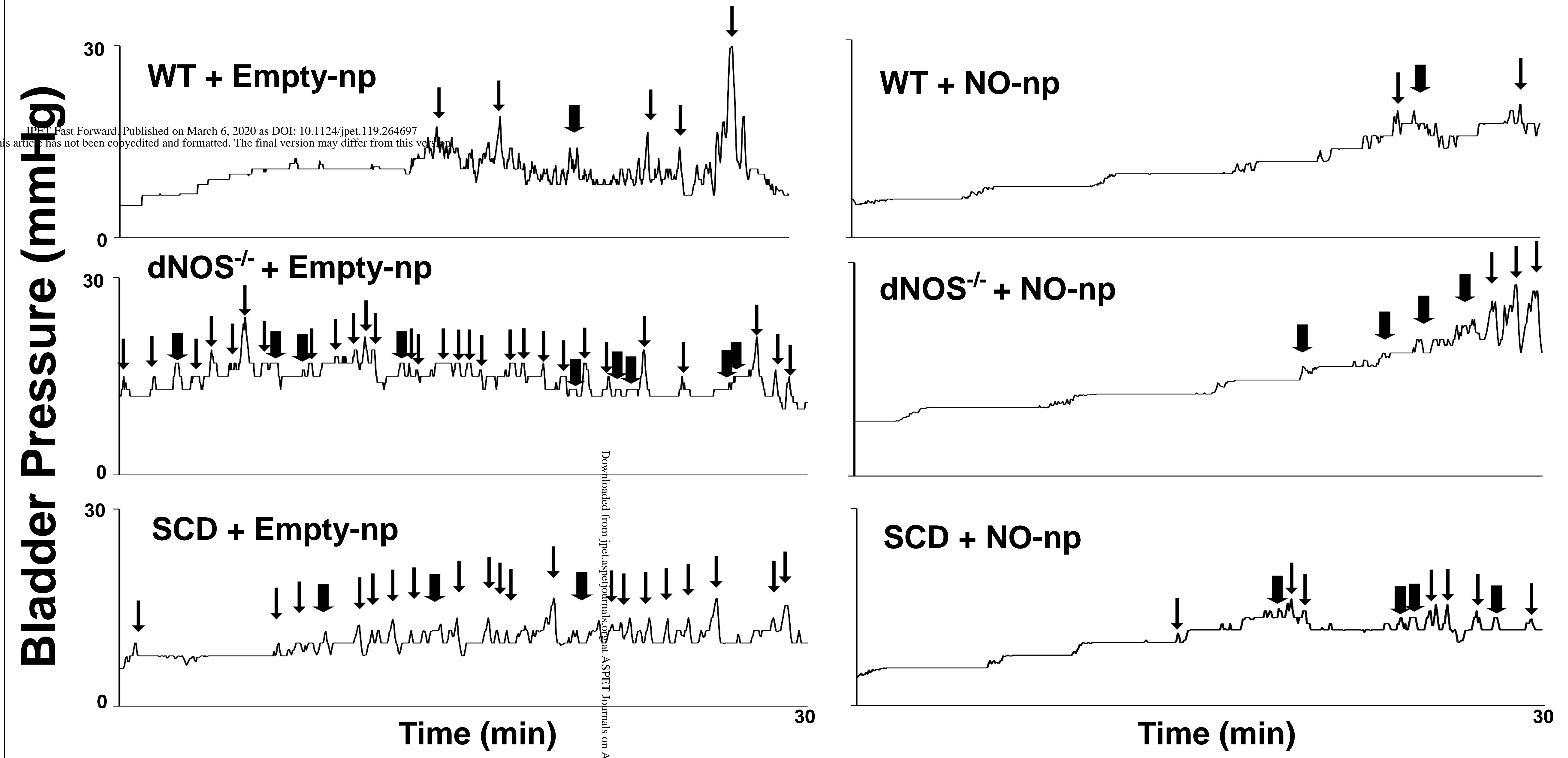


Figure 1. Representative cystometrogram traces in WT + Empty-np, WT + NO-np, dNOS$^{-/-}$ + Empty-np , dNOS$^{-/-}$ + NO-np, SCD + Empty-np, and SCD + NO-np. dNOS-/- and SCD mice exhibit unstable bladder contractions. Thick arrows indicate non-voiding contractions, thin arrows indicate voiding contractions.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

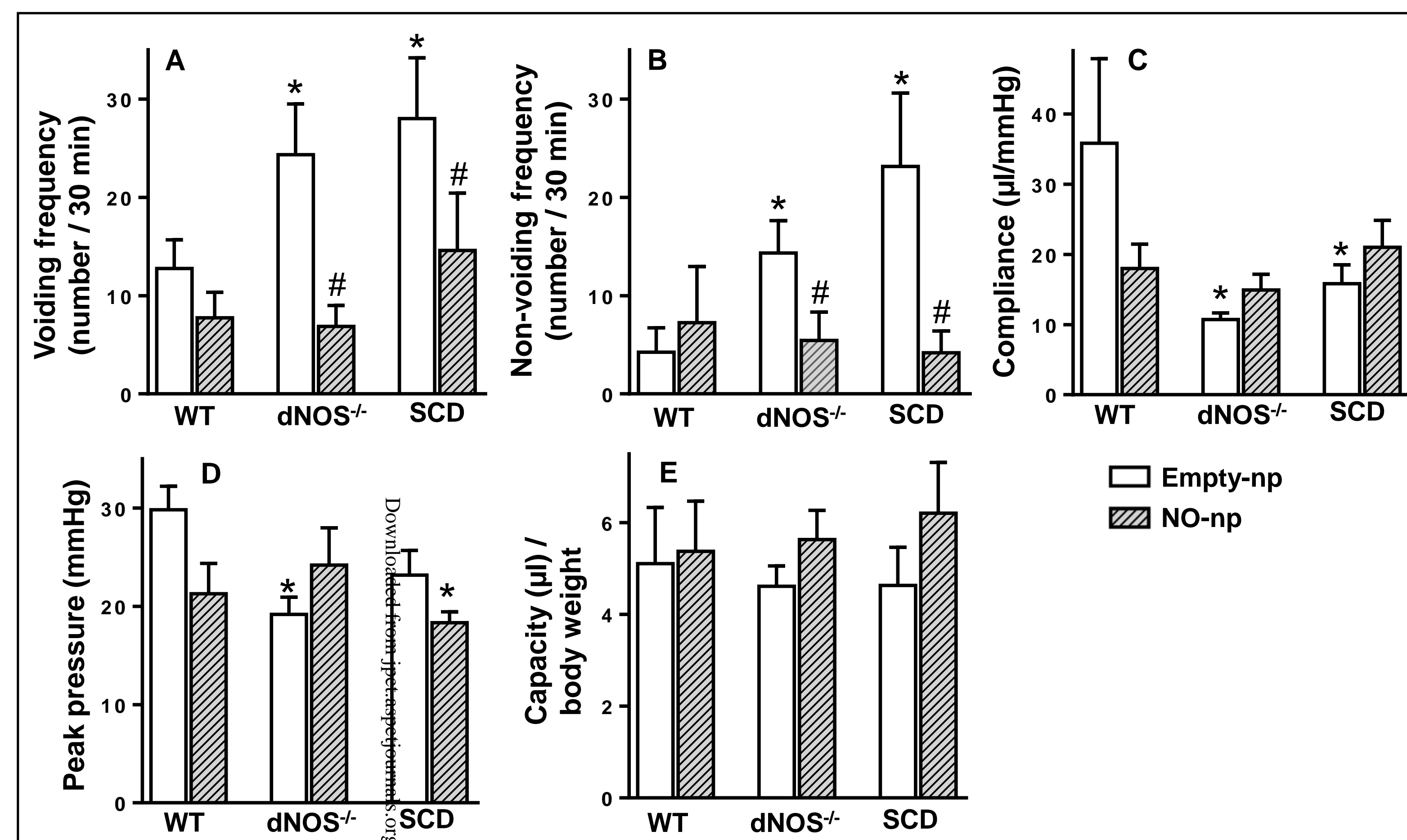


Figure 2. Effect of NO-np on cystometric parameters (voiding frequency [A], non-voiding frequency [B], compliance [C], peak pressure [D], and capacity [E]) in WT, $dNOS^{-/-}$, and SCD mice. Each bar represents the mean ± SEM. *P< 0.05 versus WT, #P< 0.05 versus Empty-np. n= 4-9

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.

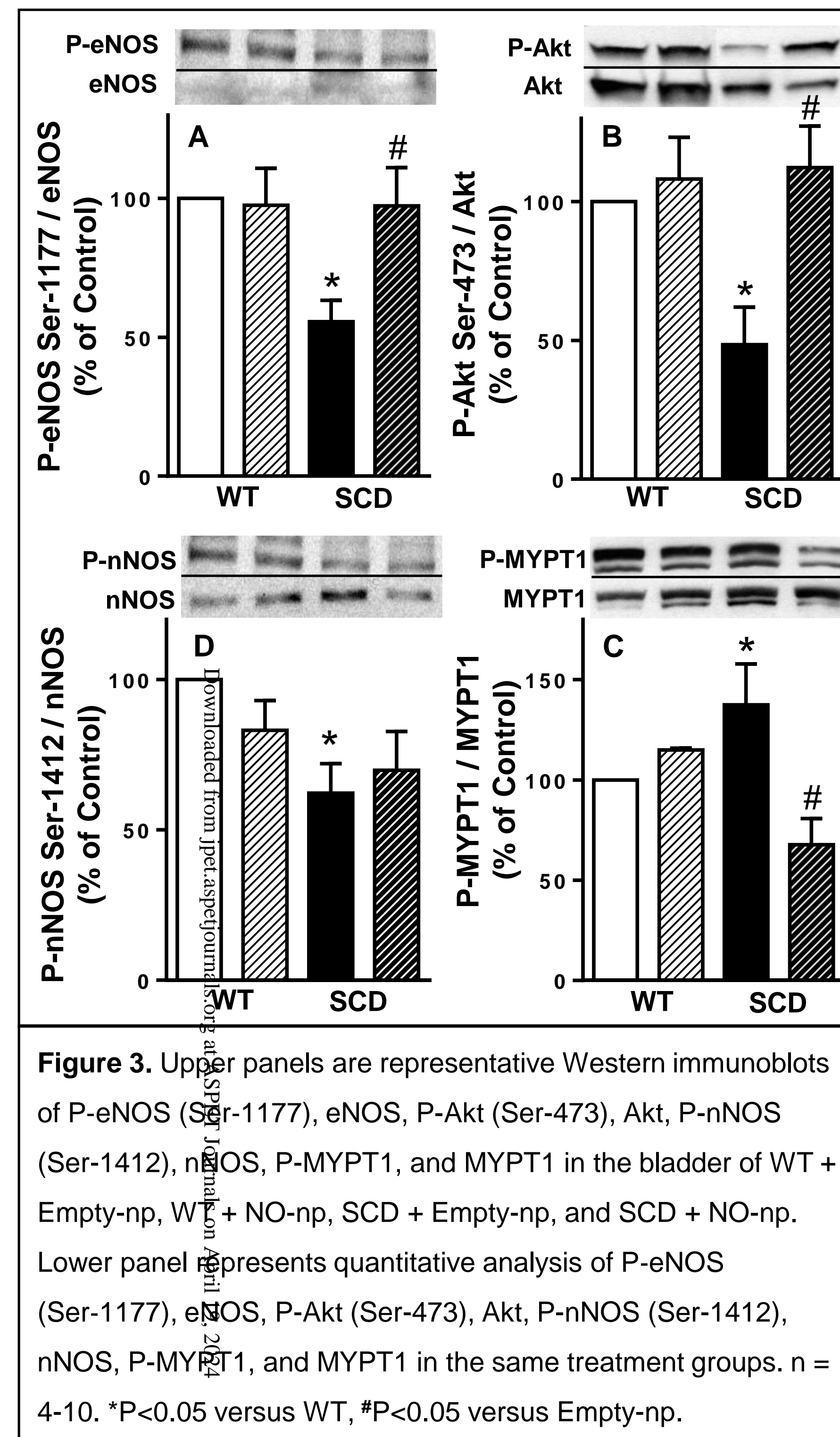
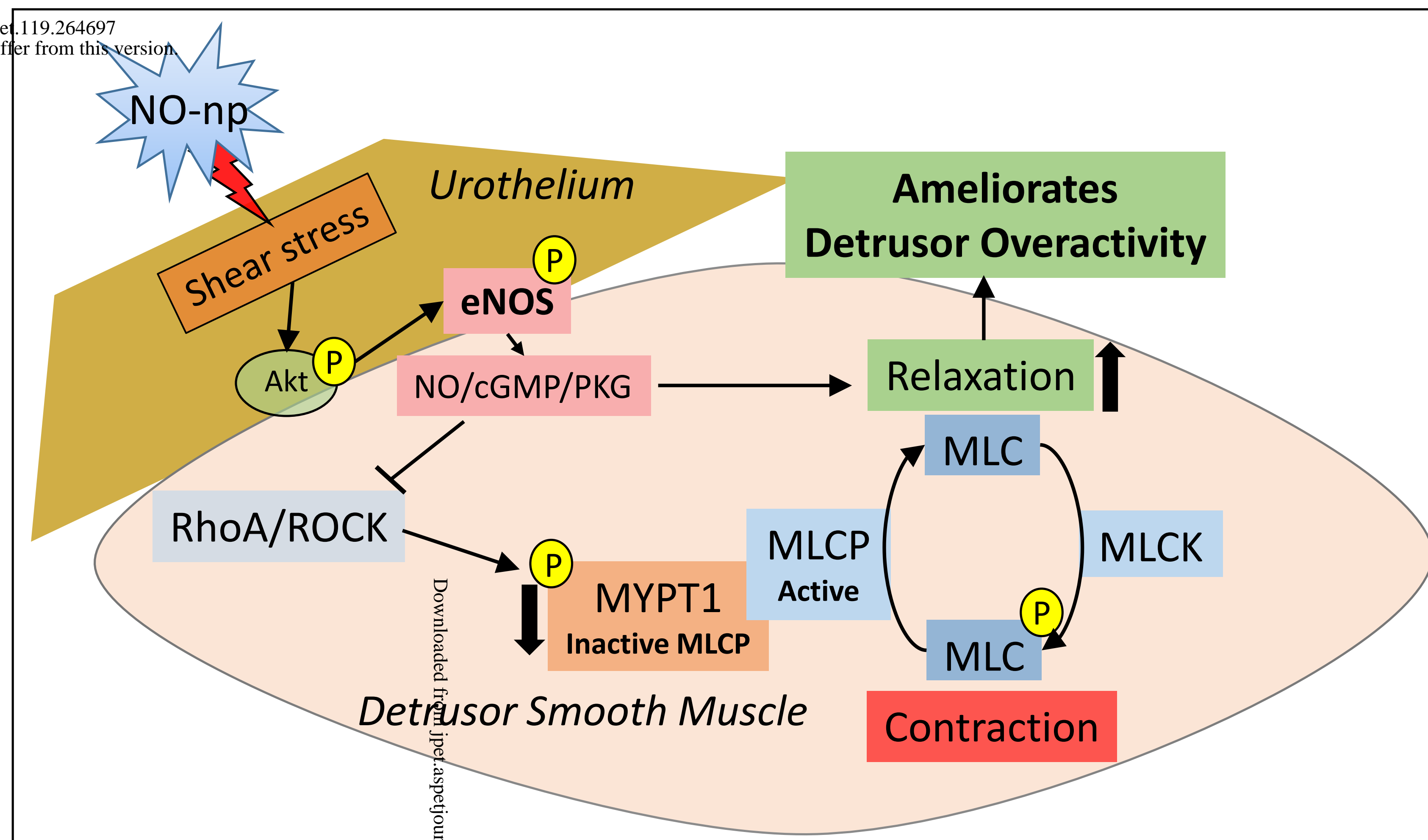


Figure 3. Upper panels are representative Western immunoblots of P-eNOS (Ser-1177), eNOS, P-Akt (Ser-473), Akt, P-nNOS (Ser-1412), nNOS, P-MYPT1, and MYPT1 in the bladder of WT + Empty-np, WT + NO-np, SCD + Empty-np, and SCD + NO-np. Lower panel represents quantitative analysis of P-eNOS (Ser-1177), eNOS, P-Akt (Ser-473), Akt, P-nNOS (Ser-1412), nNOS, P-MYPT1, and MYPT1 in the same treatment groups. n = 4-10. *P<0.05 versus WT, #P<0.05 versus Empty-np.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 22, 2024

JPET Fast Forward. Published on March 6, 2020 as DOI: 10.1124/jpet.119.264697
This article has not been copyedited and formatted. The final version may differ from this version.



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 4. Proposed mechanism of NO-np action on amelioration of detrusor overactivity in the SCD mouse
The molecular mechanism underlying detrusor overactivity in the SCD mouse involves the reduction in Akt/eNOS activities (reduced phosphorylation of Akt on Ser-473 and eNOS on Ser-1177) and increased RhoA/ROCK contractile pathway (increased phosphorylation of MYPT1, a regulatory subunit of MLCP, on Thr-696). Increased local NO by NO-np treatment increases blood flow and shear forces on the urothelium, activates Akt/eNOS (Ser-1177) phosphorylation, and promotes NO release and relaxation of the detrusor smooth muscle. Increased NO further downregulates the contractile ROCK pathway activity in the bladder by decreasing phosphorylation of MYPT-1; this activates MLCP, which dephosphorylates the MLC and initiates relaxation of detrusor smooth muscles. Normalized Akt/eNOS and RhoA/ROCK activities ameliorate detrusor overactivity.
Abbreviations: Akt = protein kinase B; eNOS = endothelial nitric oxide synthases; MLC = myosin light chain; MLCK = myosin light chain kinase; MLCP = myosin light chain phosphatase; MYPT1 = myosin phosphatase target subunit 1; NO-np = NO-releasing nanoparticles; P = phosphorylation; RhoA = ras homolog gene family, member A; ROCK = rho-associated kinase.