Sustained nitric oxide (NO)-releasing compound reverses dysregulated NO signal transduction in priapism

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ABSTRACT We evaluated the therapeutic potential of a sustained nitric oxide (NO)-releasing compound to correct the molecular hallmarks and pathophysiology of priapism, an important but poorly characterized erectile disorder. 1,5-Bis-(dihexyl-N-nitrosoamino)-2,4-dinitrobenzene (C6´) and an inactive form of the compound [1,5-bis(dihexylamino)-2,4-dinitrobenzene (C6)] were tested in neuronal cell cultures and penile lysates for NO release (Griess assay) and biological activity (cGMP production). The effect of local depot C6´ or C6 was evaluated in mice with a priapic phenotype due to double neuronal and endothelial NO synthase deletion (dNOS−/−) or human sickle hemoglobin transgenic expression (Sickle). Changes in NO signaling molecules and reactive oxygen species (ROS) surrogates were assessed by Western blot. The physiological response after C6´ treatment was assessed using an established model of electrically stimulated penile erection. C6´ generated NO, increased cGMP, and dose dependently increased NO metabolites. C6´ treatment reversed abnormalities in key penile erection signaling molecules, including phosphodiesterase type 5, phosphorylated endothelial nitric oxide synthase, and phosphorylated vasodilator-stimulated phosphoprotein. In Sickle mice, C6´ also attenuated the increased ROS markers gp91phox, 4-hydroxynonenal, and 3-nitrotyrosine. Finally, C6´ corrected the excessive priapic erection response of dNOS−/− mice. Exogenous sustained NO release from C6´ corrects pathological erectile signaling in mouse models of priapism and suggests novel approaches to human therapy.—Lagoda, G., Sezen, S. F., Hurt, K. J., Cabrini, M. R., Mohanty, D. K., Burnett, A. L. Sustained nitric oxide (NO)-releasing compound reverses dysregulated NO signal transduction in priapism. FASEB J. 28, 76–84 (2014). www.fasebj.org

Key Words: sickle cell • oxidative stress • NO bioavailability

Nitric oxide (NO) is the primary mediator of penile smooth muscle relaxation, leading to normal erectile function (1). NO is released from the cavernosal nerve fibers intercalated throughout the corpus cavernosum and from endothelial cells lining the cavernosal lacunae. Neuronal nitric oxide synthase (nNOS) is primarily responsible for initiation of normal erection, whereas both nNOS and endothelial nitric oxide synthase (eNOS) help maintain normal sustained penile erection (2, 3). NO acts primarily by stimulating production of cyclic guanosine monophosphate (cGMP) via guanylate cyclase activity in cavernosal smooth muscle cells. The NO-cGMP signal is terminated by phosphodiesterase type 5 (PDE5) hydrolysis of cGMP in the penis (4).

Priapism is a condition of abnormally prolonged supraphysiological erection, devoid of sexual purpose and with episodes often lasting longer than 4 h (5). Men with sickle cell disease (SCD) are particularly at risk, with up to 45% of men with SCD developing a urological emergency (6). The pathophysiology of priapism is now thought to be due to dysfunctional NO signaling and not merely a consequence of thrombosis and vascular blockage (7, 8). Recent animal studies showed that mice with eNOS deletion (eNOS−/−) or combined nNOS and eNOS double gene deletion (dNOS−/−) demonstrate a priapic phenotype (9, 10).

Abbreviations: 4-HNE, 4-hydroxynonenal; ANOVA, analysis of variance; AUC, area under the curve; C6, 1,5-bis-(dihexylamino)-2,4-dinitrobenzene; C6´, 1,5-bis-(dihexyl-N-nitrosoamino)-2,4-dinitrobenzene; cGMP, cyclic guanosine monophosphate; CN, cavernous nerve; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DMAC, N,N-dimethylacetamide; dNOS−/−, nNOS and eNOS double gene deletion; ED, erectile dysfunction; EIA, enzyme immunoassay; eNOS, endothelial nitric oxide synthase; Hemi, hemizygous; ICP, intracavernosal pressure; MAP, mean arterial pressure; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; NT, 3-nitrotyrosine; PDE5, phosphodiesterase 5; p-eNOS, phosphorylated endothelial nitric oxide synthase; p-VASP, phosphorylated vasodilator-stimulated phosphoprotein; ROS, reactive oxygen species; SCD, sickle cell disease; Sickle, sickle hemoglobin transgenic expression, SIN-1, 3-morpholinosydnonimine; THF, tetrahydrofuran; WT, wild type

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Sickle hemoglobin transgenic expression (Sickle) mice also develop a priapism phenotype (10–13), and, as in humans, this appears to be due to reduced NO bioavailability (14, 15). eNOS activity and phosphorylation at serine 1177 (calcium-independent eNOS activation) are decreased in the penile tissue from all three of these mutant mice, leading to local down-regulation of PDE5 in cavernosal tissue (15). Chronic down-regulation of PDE5 predisposes to priapism because the initial NO release from the nerve endings results in production of cGMP that is not metabolized and accumulates in cavernosal smooth muscle to cause persistent vasodilation of the corpora cavernosa, leading to sustained erection (16). Decreased PDE5 potentiates the cGMP signal and dramatically prolongs NO-cGMP-dependent penile erection.

Oxidative/nitrosative mechanisms that decrease endothelial NO production in the penis and therefore down-regulate PDE5 may contribute to the pathophysiology of priapism (17). Another important mechanism for decreased NO is scavenging by reactive oxygen species (ROS). In SCD, several vasculopathies occur due to oxidative stress from hemolysis. Hemolysis releases hemoglobin into the plasma where it can bind NO (18). In Sickle mice, oxidative stress contributes to cavernosal tissue damage and endothelial dysfunction via increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit expression and eNOS uncoupling (19, 20). The NADPH oxidase subunits gp91phox, p47phox, and p67phox are all increased in the penises of these mice, and oxidative stress markers increase in their cavernosal tissue, including protein carbonylation and nitrotyrosinylation (19). Priapic Sickle mice show increased ROS markers compared with those shown by control mice without priapism (11). Another marker of oxidative stress is lipid peroxidation, and 4-hydroxynonenal (4-HNE), an oxidized lipid product that forms covalent adducts with cellular proteins, is also increased in the Sickle penis (19).

Because decreased NO bioavailability leads to dysregulated downstream signaling, we have developed exogenous NO donor compounds. Administration of NO donors relaxes penile cavernosal tissue to produce erection (21, 22), but the limited availability of high-quality NO donors has inhibited progress. Commercially available sustained NO donors remain sparse. Recently, a new nitrosamine-based NO donor, 1,5-bis(dihexyl-N-nitrosoamino)-2,4-dinitrobenzene (C6), was synthesized by Dr. Dipil Mohanty (Central Michigan University, Mt. Pleasant, MI, USA), and the reaction procedures are described in brief below (23). N,N-Dimethylacetamide (DMAC), 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and hexylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Toluene was obtained from Fisher Scientific (Jessup, MD, USA). The commercial NO donor 3-morpholinosydnonimine (SIN-1) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Preparation of C6 and C6′

For the synthesis of the inactive compound C6, hexylamine (1.01 g, 0.01 mol), DFDNB (1.020 g, 0.005 mol), anhydrous potassium carbonate (2.20 g, excess), DMAC (20 ml), and toluene (20 ml) were added to a reaction vessel. The reaction mixture was heated to 60°C for 30 min, and then the temperature was gradually raised to 150°C over a period of 2 h. Water, the by-product of the reaction, was removed by azeotropic distillation with toluene through the Dean-Stark trap, and the reaction vessel was cooled to room temperature. Tetrahydrofuran (THF; 25 ml) was added to the reaction mixture, and the filtrate was evaporated at reduced pressure to remove all solvent. The residual was dissolved in dichloromethane, filtered and washed twice with water, dried over anhydrous magnesium sulfate, and filtered again, and the filtrate was evaporated using a rotary evaporator to remove dichloromethane. It was recrystallized from dichloromethane/hexane to yield the desired compound (62.8%). For the synthesis of the active NO-releasing compound C6′, inactive C6 (0.163 g), was dissolved in 6 ml of THF, and acetic acid (6 ml) was added to adjust the pH to 2–3. The reaction vessel was immersed in a salted ice bath. After the temperature of the solvent mixture had reached 0°C, sodium nitrite (0.12 g, molar ratio 8:1 to C6) was added, and the nitrosation reaction was carried out for 8 h in the dark. At the completion of the reaction, 4 N sodium hydroxide solution (27 ml) was added to raise the pH of the reaction mixture to 9–10. The mixture was extracted twice with dichloromethane (40 ml+20 ml), followed by 10% aqueous sodium bicarbonate solution (20 ml). The organic layer was dried over anhydrous magnesium sulfate and filtered, and the filtrate was evaporated under reduced pressure to yield a yellow solid. The product was stored in sealed vials under an argon atmosphere at 4°C. The degree of nitrosation was determined to be ~70% using infrared spectroscopy. Infrared spectra (pure) were acquired with a Magna IR-560 spectrometer ( Nicolet Instrument Corp., Madison, WI, USA). 1H and 13C NMR spectra were obtained using a Varian Mercury Plus-300 MHz NMR instrument ( Varian Medical Systems Inc., Palo Alto, CA, USA) using deuterated trichloromethane as the solvent.

Reagents and chemicals

C6′ and an inactive form of the compound, 1,5-bis(dihexyl-N-nitrosoamino)-2,4-dinitrobenzene (C6), were synthesized by Dr. Dipil Mohanty (Central Michigan University, Mt. Pleasant, MI, USA), and the reaction procedures are described in brief below (23). N,N-Dimethylacetamide (DMAC), 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and hexylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Toluene was obtained from Fisher Scientific (Jessup, MD, USA). The commercial NO donor 3-morpholinosydnonimine (SIN-1) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Materials and Methods

The NADPH oxidase subunits gp91phox, p47phox, and p67phox are all increased in the penile tissue from all three of these mutant mice. Microwave irradiation was carried out for 8 h in the dark. At the completion of the reaction, 4 N sodium hydroxide solution (27 ml) was added to raise the pH of the reaction mixture to 9–10. The mixture was extracted twice with dichloromethane (40 ml+20 ml), followed by 10% aqueous sodium bicarbonate solution (20 ml). The organic layer was dried over anhydrous magnesium sulfate and filtered, and the filtrate was evaporated under reduced pressure to yield a yellow solid. The product was stored in sealed vials under an argon atmosphere at 4°C. The degree of nitrosation was determined to be ~70% using infrared spectroscopy. Infrared spectra (pure) were acquired with a Magna IR-560 spectrometer ( Nicolet Instrument Corp., Madison, WI, USA). 1H and 13C NMR spectra were obtained using a Varian Mercury Plus-300 MHz NMR instrument ( Varian Medical Systems Inc., Palo Alto, CA, USA) using deuterated trichloromethane as the solvent.

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Transgenic mice
Adult male dNOS−/− mice (6–10 mo old) were originally developed on a BL6/129 hybrid background strain and backcrossed 12 generations on a C57BL/6 strain (9, 10, 24, 25). Wild-type (WT; C57BL/6) mice were used as controls (The Jackson Laboratory, Bar Harbor, ME, USA). Transgenic Sickle mice (7–10 mo old) with total knockout of mouse hemoglobin genes (Hba and Hbb) and insertion of a single transgene expressing entirely human sickle hemoglobin genes (HBA and HBB) were used (26). An in-house breeding colony was created to generate animals for this study, and age-matched hemizygous (Hemi) littermates that shared the same human transgene and mouse α-globin knockout but retained the expression of one copy of the mouse β-globin gene were used as controls (12, 19). Genotyping was performed by Transnetyx, Inc. (Cordova, TN, USA). All animal studies were conducted in accordance with the ethical standards of the Johns Hopkins University School of Medicine Guidelines for the Care and Use of Animals.

Primary cortical neuronal culture
Rat primary cortical neurons were prepared from 17- to 18-d-old embryonic Sprague-Dawley rats as described previously (27, 28) and grown for 10–14 d on poly-d-lysine-coated 6-well plates. Phenol-free medium was added, and cells were incubated for 14 h with 10 mg/well of C6 or C6, SIN-1 (20 μM) or an equal volume of vehicle (ethanol) was applied for 15 min before cell harvest as positive and negative controls, respectively. The cells and medium were collected and centrifuged (5000 g for 5 min), and the supernatant was assayed for total nitrite (NO3). Neuronal cells were lysed in 0.1 M HCl + 100 μM sodium metatungstate, sonicated, centrifuged (16,000 g for 20 min), and assayed for cGMP. No abnormal cell morphology was observed with C6’ or C6 treatment; axonal processes were not retracted, and cell adherence to the culture plate was well-maintained, similar to our prior observations with neuron-like PC12 cells (23).

Griess reaction
To measure NO release in medium, modified Griess reagent was used (Sigma-Aldrich). To measure NO release in penile homogenates or serum, a separate Griess reaction measuring NO to nitrate (NO3) was performed using a commercially available kit (Oxford Biomedical Research, Rochester Hills, MI, USA) as described previously (23). This kit uses metallic cadmium for quantitative conversion of NO3 to NO2 before quantitation of NO2 using the Griess assay, therefore providing for accurate determination of total NO (NOx) production. For Griess assays, absorbance was measured at 540 nm using a 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), and NO3 concentrations were determined using a NO2 standard curve, supplied by the manufacturer.

cGMP assay
Cellular cGMP was measured using a commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical), according to the manufacturer’s protocol. Supernatants were diluted in EIA buffer and added to a 96-well plate along with an EIA standard solution for the standard curve. Then 50 μl each of the cGMP acetylcholinesterase tracer and cGMP EIA antisera was added to each well and incubated overnight. On the following day Ellman’s reagent was added to each well, and the absorbance was measured at 420 nm.

dNOS−/− penile homogenate treatment with C6’
At room temperature, 15 or 30 mg of C6’ was added to dNOS−/− penile tissue homogenates. Penile tissues were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM β-glycero-phosphate; 1 mM Na3VO4; and 1 μg/ml leupeptin), and protein concentrations were determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). The supernatant was shaken briefly with a Vortex mixer and incubated for 2 h. SIN-1 and ethanol were positive and negative NO controls, respectively. Samples were centrifuged (2000 g for 5 min) to remove insoluble material, and NOx in the supernatant was measured.

C6’ in vivo administration
A midline incision was made anterior to the scrotum to identify the bulbo-spongious muscle at the base of the penis, and a small cavity was created (Supplemental Fig. S1). Then 10 mg of C6’ or C6 was deposited and sutured in place with a subcutaneous closure. The skin was sutured, and the animal was allowed to recover for 1 wk. For dNOS−/− experiments, mice were divided into 4 groups: WT + saline, dNOS−/− + saline, dNOS−/− + C6, and dNOS−/− + C6’. One week after treatment, penises and lungs were collected for Western blot analysis of PDE5, and serum was collected for NO measurement. For Sickle experiments, mice were divided into 4 groups: Hemi + C6, Hemi + C6’, Sickle + C6, and Sickle + C6’. One week after treatment, penises were collected for Western blot analysis of PDE5, p-cNOS, total eNOS, p-VASP, total VASP, 4-HNE, and gp91phox.

Western blot analysis
Penile tissues were homogenized as described above, and supernatants (30 or 60 μg total protein) were run on 4–20% Tris-HCl gels (Bio-Rad Laboratories), transferred to a polyvinylidene fluoride membrane, and incubated overnight at 4°C with the following antibodies: polyclonal PDE5 and monoclonal NT (1:1000; Abcam Inc., Cambridge, MA, USA), polyclonal anti-p-cNOS (Ser-1177, 1:450; Cell Signaling Technology, Beverly, MA, USA), polyclonal anti-p-VASP (Ser-239, 1:1000; Cell Signaling Technology), polyclonal anti-4-HNE antibody (1:2000; Alpha Diagnostic International, San Antonio, TX, USA), and monoclonal anti-gp91phox (1:1000; BD Transduction Laboratories, San Diego, CA, USA). Membranes used for p-cNOS and p-VASP analyses were stripped and probed with antibodies for the index proteins: mouse anti-cNOS (1:1000; BD Transduction Laboratories), or rabbit anti-VASP (1:1000; Cell Signaling Technology). Other membranes were normalized to β-actin (1:10,000; Sigma-Aldrich). Blots were scanned and quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Quantified densitometry results were normalized to the control group and then set as a ratio to β-actin quantified densitometry results, also normalized to the control group and plotted on the same gel, making the control group 1 arbitrary unit.

Physiological erection studies
In vivo erectile function in response to stimulation of the cavernous nerve (CN) was studied in WT and dNOS−/− mice.
analyses). For Western blots, we used a modified followed by a Newman-Keuls multiple comparison (for the were performed using 1-way analysis of variance (ANOVA), compare the experimental groups with the normalized control ratio, and to evaluate among the experimental exposure the prostate where the right major pelvic ganglion and CN were identified posterolateral to the prostate. For electrically stimulated penile erections, a bipolar electrode attached to an S48 stimulator (Grass Instruments, Quincy, MA, USA) was placed around the CN, and stimulation parameters were 1 V at a frequency of 16 Hz with square-wave duration of 5 ms. ICP was recorded using the DI-190 system (Dataq Instruments, Akron, OH, USA) from the beginning of the experiment when the cannula was inserted into the crura continuously until the animal was euthanized. Erectile function was represented by the maximal ICP response during electrical stimulation, the baseline ICP response before electrical stimulation [prestimulated area under the curve (AUC)] and the baseline ICP response after stimulation (poststimulated AUC). Prestimulated AUC was calculated for 3 min from the time of cannula insertion to the start of electrical stimulation. Electrical stimulation of the CN was for 60 s. Poststimulated AUC was calculated for 3 min starting at the end of the electrical stimulation parameters. Results were analyzed using the MATLAB program (MathWorks, Natick, MA, USA). The carotid artery was cannulated with PE-10 tubing and attached to a pressure transducer to measure mean arterial pressure (MAP).

Statistical analyses

Data are expressed as means ± SEM. Statistical analyses were performed using 1-way analysis of variance (ANOVA), followed by a Newman-Keuls multiple comparison (for the NO and cGMP assays) or by a Student’s t test (for ICP analyses). For Western blots, we used a modified t test to compare the experimental groups with the normalized control ratio, and to evaluate among the experimental groups, we used a 1-way ANOVA followed by Tukey’s multiple comparison test. A value of $P < 0.05$ was considered significant.

RESULTS

Effect of C6’ on NO release and cGMP production in vitro

To verify bioactive NO release by C6’, we treated rat cortical neuronal cultures in vitro with C6’ (Fig. 1A). Treatment with the positive control NO donor SIN-1 for 20 min caused a 6-fold increase in NO$_2$ compared with that for ethanol or inactive C6 (P<0.05). NO$_2$ was increased >30-fold in medium treated with C6’ overnight compared with C6 treatment (P<0.001). NO stimulation of second-messenger cGMP production was also measured in neuronal cells treated with SIN-1 or C6’ (Fig. 1B). SIN-1 significantly increased cGMP levels compared with those for ethanol or C6 (P<0.05). C6’ significantly increased cGMP levels as well (P<0.01). To test whether C6’ releases NO in a complex tissue homogenate, we added C6’ to penile dNOS$^{-/-}$ tissue homogenates (Fig. 1C). C6’ (15 and 30 mg) led to increased NO$_x$ formation compared with that for ethanol in a dose-dependent manner (P<0.05). These data confirm that C6’ serves as a physiological source of NO release and potently affects cGMP production.

Effect of C6’ on PDE5 expression in dNOS$^{-/-}$ mice in local and distant targets

Because decreased NO signaling leads to lower PDE5 in the penis and is associated with priapism (10), we examined the effect of restoring basal NO production with C6’ treatment in dNOS$^{-/-}$ mice. PDE5 expression was significantly decreased in penises of the dNOS$^{-/-}$ + saline and dNOS$^{-/-}$ + C6 groups compared with WT group values (P<0.05; Fig. 2A). Treatment with C6’ did not cause any adverse effects in the animals and significantly increased PDE5 expression levels compared with those for the dNOS$^{-/-}$ + saline and dNOS$^{-/-}$ + C6 groups (P<0.05). NO can be transferred via red blood cell heme to influence whole-body NO signaling (29); thus, to evaluate whether locally administered C6’ increases systemic or whole-body NO$_x$, serum levels

Figure 1. NO$_x$, NO$_2$, and cGMP levels are increased after C6’ exposure in vitro. A) Treatment with SIN-1 for 20 min caused a significant 6-fold increase in NO$_2$ compared with that for ethanol or C6 treatment. After C6’ treatment, NO$_2$ levels were increased compared with those after ethanol or C6 treatment. B) C6’ treatment also generated cGMP elevations compared with those for ethanol and C6 treatment. C) In penile tissue homogenates, NO$_x$ was significantly increased after C6’ incubation (15 or 30 mg) compared with that for ethanol treatment. Furthermore, C6’ dose dependently produced NO$_x$ increases such that penile homogenates treated with 30 mg of C6’ produced more NO$_2$ than homogenates treated with 15 mg. Bars are means ± SEM of 3–5 mice/group. *P<0.05, **P<0.01, ***P<0.001 vs. ethanol; $^a$P<0.05, $^ab$P<0.001 vs. C6; $^b$P<0.05 vs. C6’ 15 mg.
were measured in dNOS−/− mice. There were no differences in serum NO3 levels between C6′ (24.5± 2.06) and C6 (22.2± 4.7) treatment groups. We also determined PDE5 levels in dNOS−/− lung tissue after C6′ and C6 treatment (Fig. 2B). There were no differences in PDE5 expression between C6′ (0.495± 0.09) and C6 (0.513± 0.11) treatment groups, suggesting its local effect only. Together, these data indicate that depot C6′ locally increases PDE5 expression in penile tissue without altering NO signaling in distant sites.

**Effect of C6′ on NO signaling and ROS mediators in Sickle mice**

Similar to the result in dNOS−/− mice, PDE5 expression was restored in Sickle mice with C6′ treatment compared with C6 treatment (P<0.05) and was similar to that in Hemi group mice (Fig. 3A). Given that C6′ normalized down-regulated PDE5 expression in the Sickle penis, we evaluated other altered signaling molecules in SCD. p-eNOS and p-VASP reflect NO activity and NO production, respectively. Expression levels of p-eNOS and p-VASP in the penis were significantly decreased in the Sickle + C6 group compared with Hemi group values (P<0.05; Fig. 3B, C), and treatment with C6′ normalized their expression (P<0.05). Because ROS and oxidative stress in the Sickle penis is associated with decreased NO bioavailability (11, 19), we evaluated the oxidative stress markers 4-HNE and NT as well as the NADPH oxidase subunit gp91phox with C6′ treatment. 4-HNE, NT, and gp91phox expressions were significantly higher in the Sickle + C6 group compared with Hemi group values (P<0.05; Fig. 4). C6′ treatment significantly lowered 4-HNE, NT, and gp91phox expressions, consistent with Hemi group values (P<0.05). These results confirm the previous understanding that NO signaling is altered via changes in eNOS and VASP activity in concert with heightened oxidative stress regulators in the Sickle mouse penis and suggest that C6′ reverses aberrant NO and ROS signaling.

**Erection physiology in dNOS−/− mice**

We performed physiological erection studies in WT and dNOS−/− mice to evaluate the effectiveness of C6′ in regulating the priapism phenotype. Uncontrolled erectile responses, indicated by increased prestimulated and poststimulated AUC levels, were found in dNOS−/− mice treated with the inert compound C6 (Fig. 5A). dNOS−/− mice treated with 10 mg of C6′ for 1 wk had significantly normalized prestimulated and poststimulated AUC, and levels were similar to those found in WT mice. Maximal ICP values were similar in dNOS−/− mice treated with C6 and C6′ (Fig. 5B). In WT mice, AUC and maximal ICP levels were similar among C6′ and C6 treatment groups, suggesting that C6′ does not have the same effect in a normal mouse without NOS gene deletions. To exclude the possibility that C6′ itself may affect ICP readings by affecting blood pressure, we found similar MAP in C6′-treated (181.1±4.7) and C6-treated (180.3±7.9) mice. Representative tracings of erectile responses show that dNOS−/− mice have unstable ICP values before and after electrical stimulation. After treatment with C6′, the exaggerated erectile response normalized (Fig. 5C).

**DISCUSSION**

We have developed a novel approach for depot administration of the slow and sustained NO-releasing compound C6′ and offer evidence for a therapeutic effect...
of this technique in correcting the molecular abnormalities seen with chronic NO deficiency. NO is released from C6′ in a dose-dependent manner, and C6′ stimulates cGMP production, confirming biological activity. PDE5 expression in the penis is down-regulated in 2 mouse strains with decreased NO activity, leading to a priapism phenotype, dNOS−/− and Sickle mice, and treatment with C6′ restored normal PDE5 expression. In the Sickle mouse penis, p-eNOS and p-VASP, markers of eNOS activation and NO production, respectively, were decreased, but 1 wk of depot C6′ treatment restored normal protein levels. Similarly, penile expression of the oxidative stress markers 4-HNE, NT, and the NADPH oxidase subunit gp91phox reverted to normal/control levels after C6′ treatment in Sickle mice. In an in vivo physiological model of erectile function using electrical stimulation of the CN, dNOS−/− mice display uncontrolled baseline and poststimulation ICP with

Figure 3. C6′ treatment in Sickle mouse penis corrects aberrant PDE5 and NO signaling. A) PDE5 expression was significantly lower in the penis of the Sickle + C6 group compared with both Hemi group values. C6′ treatment significantly restored PDE5 expression in the Sickle mouse penis. B) p-eNOS expression was significantly lower in the penis of the Sickle + C6 group compared with both Hemi group values. C6′ treatment significantly restored p-eNOS expression in the Sickle mouse penis. C) p-VASP expression was significantly lower in the penis of the Sickle + C6 group compared with both Hemi group values. C6′ treatment significantly restored p-VASP expression in the Sickle mouse penis. Top panels: representative immunoblots; bottom panels: densitometry ratios. Bars represent means ± SEM of 6–8 mice/group. A single band for PDE5 was normalized to β-actin. Doublet bands for p-VASP (~48 and 50 kDa) were normalized to total VASP, and a single band for p-eNOS was normalized to total eNOS. *P < 0.05 vs. both Hemi groups; †P < 0.05 vs. Sickle + C6′.

Figure 4. Attenuation of heightened oxidative stress after C6′ treatment in Sickle mouse penis. A) gp91phox expression was significantly higher in the penis of the Sickle + C6 group compared with both Hemi group values. C6′ treatment significantly attenuated this increase in the Sickle mouse penis. B) 4-HNE expression was also significantly higher in the penis of the Sickle + C6 group and was significantly attenuated with C6′ treatment. C) NT expression was also significantly higher in the penis of the Sickle + C6 group and was significantly attenuated with C6′ treatment. Top panels: representative immunoblots. Bottom panels: densitometry ratios. Bars represent means ± SEM of 6–8 mice/group. A single band for gp91phox or the whole lane of total protein for 4-HNE and NT was normalized to β-actin. *P < 0.05 vs. both Hemi groups; †P < 0.05 vs. Sickle + C6′.
irregular priapic increases in cavernosal pressure. Treatment with C6' for 1 wk restored normal ICP responses similar to those in WT animals. WT mice, on the other hand, were unaffected by C6' application. Because depot C6' does not increase serum NOx or change PDE5 expression in distant tissues (lung), the effect is probably local and may not influence overall NO homeostasis. These experiments suggest that a sustained NO donor delivered by a novel approach for depot administration can reverse the priapism phenotype due to abnormal NO signaling or increased oxidative stress by correcting molecular alterations seen in dNOS−/− and Sickle mice.

Down-regulation of the NO/cGMP/PDE5 pathway in the penis occurs with chronically reduced NO production, as we have shown in prior studies. Both eNOS−/− and dNOS−/− mice have a priapism-like phenotype due to down-regulated PDE5 activity and increased cGMP accumulation in penile tissue (3, 10). Priapic behavior can be explained by a physiologically relevant supersensitization to cGMP with CN stimulation. As a result of chronically decreased PDE5 expression, cGMP generated with neuronal stimulation produces uninhibited cavernosal smooth muscle relaxation and prolonged erection (10). Similar defects in the NO signaling pathway have recently been identified in animal models of SCD (12, 30), implying that deficient NO signaling may mediate human disorders of aberrant smooth muscle relaxation and neurovascular physiology. The pathogenesis of impaired NO bioavailability in SCD includes NO scavenging by cell-free plasma hemoglobin, decreased plasma L-arginine substrate, increased ROS causing oxidative stress, and damaged endothelial cells (30). In SCD, decreased PDE5 and eNOS expression are observed in the human (31), and endothelial contributions of NO may be decreased (15). Studies in Sickle mice show that decreased PDE5 and eNOS activities in the penis lead to priapism (10, 15). The dNOS−/−/mice used in our studies are a convenient and less costly model for investigating the pathophysiologic consequences of reduced NO production and generally represent the same pathological processes we have identified in Sickle mice. Although the initiating events leading to decreased NO production in the dNOS−/− and Sickle mice are clearly different (i.e., genetic knockout vs. chronic endothelial damage), the resulting deficiency of NO leads to the same priapism phenotype, and, to our surprise, supplying low levels of local NO with depot polymer appears to correct molecular changes in both animal models. Further studies on the therapeutic mechanism of C6' in Sickle mice are ongoing in our laboratory.

All of the components of altered NO signaling in our animal models, including NO production (p-eNOS), cGMP metabolism (PDE5), and downstream effector activation (p-VASP), are normalized by restoring low-dose, sustained-release exogenous NO. Although increased substrate activation and induction of metabolic enzymes can be easily explained by the increased supply of NO (32), the unexpected increase in p-eNOS suggests a secondary beneficial effect of C6'. Our prior work in mice showed that increased expression of eNOS by adenoviral transfection can correct downstream abnormalities in eNOS−/− mice (10), but our experiments here show that maintaining NO can increase synthetic enzyme activity as well. This finding has important therapeutic implications for disorders of endothelial function in which eNOS levels may be decreased overall. Supplying exogenous NO may be as advantageous and less toxic than transfecting exogenous NOS enzymes. Decreased NO appears to alter all parts of the NO signaling cascade regardless of the underlying cause, and C6' normalizes the entire pathway.

The role of ROS and oxidative stress mediating priapism has been described recently (11, 19, 31). Activated endothelial cell NADPH oxidase has been implicated in ROS production in the cerebral vasculature of Sickle mice and is a key mediator of ROS and oxidative damage in the Sickle mouse penis (19, 33). ROS produced by NADPH oxidase causes eNOS uncoupling and increased monomerization of the enzyme, leading to endothelial dysfunction (19, 34). Consequently, altered endothelium-dependent vascular relaxation has been associated with decreased bioavailability of NO· through ROS in animal models of hypertension and diabetes. For this study, we examined the effect of C6' on oxidative stress markers (gp91phox, 4-HNE, and NT) previously shown by our laboratory to be up-regulated in Sickle mice (19). We confirmed increased gp91phox, 4-HNE, and NT in Sickle mice. It is possible that increased NADPH oxidase
activity and superoxide production causes increased 4-HNE (via lipid peroxidation) and peroxynitrite (in combination with NO) formation. Surprisingly, C6’ administration restores control levels of all 3 of these markers in Sickle mice, despite increased local release of very reactive NO. NO donors have been reported to depress NADPH oxidase activity (35, 36), which could account for our results, although the antioxidant properties of NO under some conditions are equally plausible (37, 38). Our results in Sickle mice show that C6’ is beneficial in normalizing harmful ROS production in the penis and suggest that it can be beneficial not only for erectile disorders related to altered NO signaling but also for those related to increased ROS and endothelial dysfunction. We are performing additional studies to evaluate whether C6’ influences other oxidative/antioxidative regulators, such as xanthine oxidase, eNOS uncoupling, superoxide dismutase, and glutathione peroxidase.

Small molecule therapies for endothelial vasculopathy have been studied in both animal and human models (39). The clinical utility of PDE5 inhibitors (e.g., sildenafil) to treat erectile dysfunction (ED) is well known. In ED, reduced NO production or responsiveness with normal PDE5 levels leads to decreased cGMP and reduced sexual function. Because PDE5 levels are not altered, cGMP is produced at a lower level and is quickly metabolized, and no NO-dependent response is produced. In priapism, however, the combination of chronic NO deficiency, increased ROS, and intact neuronal NO signaling, creates a superresponse to the initiation phase of NO production, and sustained cGMP levels lead to prolonged elevations in ICP. Priapism then represents an erectile disorder distinct from conventional ED, both in its clinical presentation and pathophysiology, and its significance is underscored by its frequency in certain clinical populations (as many as 40% of men with SCD are affected) and its predictable complications of penile ischemia (cavernosal tissue dysfunction and fibrosis; ref. 40). Small clinical studies using PDE5 inhibitors for prevention of priapism have shown some promise (41), because they may help to increase PDE5 expression over time and thereby correct the abnormal NO metabolism. Restoration of normal NO levels, as in our study, is an attractive option because it appears to correct multiple abnormalities both upstream and downstream and may restore more durable normal function.

We previously showed that the NO donor C6’ decreases cell death in PC12 cells after exposure to the ROS compound hydrogen peroxide (23). Low levels of C6’ (0.02–0.2 mg/ml) were protective, whereas higher concentrations (2.0 mg/ml) were harmful. The potential for NO to cause positive or negative effects has been described for many years (42). Many NO donors release NO in bolus that can be toxic or affect whole-animal parameters such as blood pressure (43, 44). Our N-nitrosoamine-based C6’ controls the rate of NO release by the length of the aliphatic spacer groups between the aromatic moieties and releases NO in a slow and sustained manner without an NO burst. The sustained release occurs for up to 30 d (23). We studied just one concentration of C6’ (10 mg), estimated from our prior studies. This dose did not produce adverse effects in animals, although a full therapeutic dose-response investigation, in vitro and in vivo, will help to delineate the most effective dosing strategies. MAP in vivo was similar in mice treated with C6 or C6’, suggesting that local penile administration of C6’ did not influence blood pressure. Unfortunately, similar to most NO donors, we do not have any current in vivo data to show toxicity and biodegradability of C6’ nor do we know how it is metabolized or excreted from the body. The application of C6’ for depot treatment of human erectile disorders awaits development of a nontoxic and completely metabolized NO carrier, although these studies provide proof of principle.

The advantages of our studies include demonstration of normal NO signaling by the C6’ compound in vitro and normalization of physiological erectile function in whole-animal dNOS−/− in vivo studies. We have also used an established rodent model of human disease, Sickle transgenic mice, to show that C6’ corrects several molecular abnormalities that underlie the priapism erectile disorder. Although we have not shown physiological correction of Sickle animal priapism, the dNOS−/− mice are a reasonable proxy for molecular alterations leading to priapism due to chronic reduction of NO. Additional studies in both mice and humans will determine whether restoring normal NO signaling with long-term exogenous NO donors corrects or prevents the priapism defects seen in SCD or possibly acts synergistically with current therapies for erectile dysfunction as well. Further, we are interested in examining the effects of sustained exogenous NO on the vascular insults and thrombotic propensity seen in SCD. It is possible that supplemental NO may have positive upstream and downstream effects.

In summary, this study provides evidence that a nitrosoamine-based slow and sustained release NO donor ameliorates dysfunctional NO signal transduction systems involved in vasodilation (NO/cGMP/PDE5 signaling) and oxidative stress (lipid peroxidation and NADPH oxidase activity) in the penis and suggests potential therapies for priapism, particularly when related to SCD. SCD-related priapism therapies are lacking because the pathophysiology has just become clear. In light of the recent finding that aberrant NO signaling occurs in the penis of men with SCD, techniques that normalize NO bioavailability and restore NO signaling in the penis may be of important clinical interest.

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REFERENCES


NO-RELEASING COMPOUND REVERSES PRIAPISM

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Sustained nitric oxide (NO)-releasing compound reverses dysregulated NO signal transduction in priapism


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