Published in final edited form as:

J Sex Med. 2014 February ; 11(2): 424–430. doi:10.1111/jsm.12391.

Sildenafil Promotes eNOS Activation and Inhibits NADPH Oxidase in the Transgenic Sickle Cell Mouse Penis

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Abstract

Introduction—Sickle cell disease (SCD)-associated vasculopathy in the penis is characterized by aberrant nitric oxide and phosphodiesterase (PDE) 5 signaling, and by increased oxidative stress. Preliminary clinical trials show that continuous treatment with PDE5 inhibitor sildenafil unassociated with sexual activity decreases priapic activity in patients with SCD. However, the mechanism of its vasculoprotective effect in the penis remains unclear.

Aims—We evaluated whether continuous administration of PDE5 inhibitor sildenafil promotes eNOS function at posttranslational levels and decreases superoxide-producing enzyme NADPH oxidase activity in the sickle cell mouse penis.

Methods—SCD transgenic mice were used as an animal model of SCD. WT mice served as controls. Mice received treatment with the PDE5 inhibitor sildenafil (100 mg/kg/day) or vehicle for 3 weeks. eNOS phosphorylation on Ser-1177 (positive regulatory site), eNOS interactions with heat-shock protein 90 (HSP90) (positive regulator), phosphorylated AKT (upstream mediator of

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Conflict of Interest: The authors report no conflicts of interest.

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eNOS phosphorylation on Ser-1177), an NADPH oxidase catalytic subunit gp91(phox), and a marker of oxidative stress (4-hydroxy-2-nonenal [HNE]) were measured by Western blot.

Main Outcome Measures—Effect of continuous sildenafil treatment on eNOS posttranslational activation, NADPH oxidase catalytic subunit, and oxidative stress in the penis of the sickle cell mouse.

Results—Continuous treatment with sildenafil reversed ($P < 0.05$) the abnormalities in protein expressions of P-eNOS (Ser-1177), eNOS/HSP90 interaction, P-AKT, protein expression of gp91(phox), and 4-HNE, in the sickle cell mouse penis. Sildenafil treatment of WT mice did not affect any of these parameters.

Conclusion—Our findings that sildenafil enhances eNOS activation and inhibits NADPH oxidase function in the sickle cell mouse penis offers a vasculoprotective molecular basis for the therapeutic effect of sildenafil in the penis in association with SCD.

Keywords

eNOS Phosphorylation; AKT; HSP90; 4-HNE; Oxidative Stress

Introduction

Sickle cell disease (SCD) is an inherited disorder characterized by a single point mutation that substitutes valine for glutamic acid in the β -globin subunit of hemoglobin [1]. The vasculature is a major target of SCD, and endothelial dysfunction is a critical component of the vasculopathy accompanying this disease [2]. Endothelial dysfunction primarily results from impairment of NO bioavailability [3]. Endothelial NO deficiency in SCD is a multifactorial presentation involving decreased production of NO (due to decreased eNOS activity and arginase-mediated substrate depletion) and increased scavenging of NO (by cell-free plasma hemoglobin released during hemolysis, and by reactive oxygen species [ROS]) [4–6]. Increased ROS generation and decreased antioxidant reserves both contribute to increased oxidative stress in SCD vasculature. The enzymes xanthine oxidase, NADPH oxidase, and uncoupled eNOS are major sources of ROS [7–9].

Recurrent ischemic priapism, a disorder of non-willful, excessive penile erection, associated with erectile tissue loss and erectile dysfunction, is a highly prevalent complication of SCD [10]. Molecular mechanisms leading to priapism are incompletely understood. Recent studies demonstrated that priapism results from defective endothelial-derived NO and cGMP signaling in the penis due to decreased NOS activity [5,11–16]. In addition, oxidative stress is increased in the SCD penis due to upregulation of NADPH oxidase subunits p67^{phox}, p47^{phox}, and gp91^{phox} and eNOS uncoupling [15–18], the latter also contributing to lower production of NO. Basally low levels of eNOS and NO downstream signaling in the penis of SCD mice [5,16,18] and humans with SCD [15] are associated with decreased cGMP-specific PDE5 regulatory function, such that unchecked cGMP accumulation upon neurostimulation results in priapism [16,19–21]. Penile overproduction of adenosine [22,23] and upregulation of opiorphins [24] have also been implicated in priapism.

Preliminary clinical trials involving men with SCD demonstrated that PDE5 inhibitor sildenafil proved efficacious in reducing priapism related events [11,12]. We recently reported oxidative stress, eNOS uncoupling and NO downstream signaling as major targets of sildenafil action in the SCD mouse penis, resulting in decreased priapic activity [16]. Given the central role of endothelial NO dysregulation and oxidative stress in cavernosal vasculopathy associated with SCD, and the complexity of eNOS regulation mechanisms, we hypothesized that continuous inhibition of PDE5 with sildenafil citrate would also restore eNOS posttranslational activation and decrease NADPH oxidase in the SCD penis.

Materials and Methods

Animals

Seven- to nine-month-old sickle and WT male mice were used. Transgenic sickle mice (Sickle) express exclusively human sickle hemoglobin (HbS), generated by knockout of mouse alpha and beta globins and insertion of a single transgene that expresses human alpha and beta S globin. Sickle mice are obtained by interbreeding sickle cell males with hemizygous females in-house. Breeding pairs for Sickle mice (strain number 3342) and WT mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Genotyping was performed by Transnetyx, Inc (Cordova, TN). Male Sickle mice exhibit priapism, as demonstrated by the exaggerated erectile response both pre- and post- electrical stimulation of the cavernous nerve compared with wild type (WT) mice [16,19,21]. Aged-matched WT C57BL/6 males were used as controls because this represents the predominant background strain for the transgenic mice [25]. Sickle and WT mice were fed with the PDE5 inhibitor sildenafil citrate (100 mg/kg/day, Viagra, Pfizer) or vehicle for 3 weeks by mixing the drug into semi-soft rodent chow (Bioserve; 4–6 g/d) [26]. At the end of the treatment, flaccid penes were collected before WT and Sickle mice were sacrificed. 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.

Western Blot Analysis

Penile tissue was homogenized as described [27]. P-eNOS (Ser-1177)/eNOS and eNOS interaction with HSP90 were evaluated in penile samples partially purified for NOS enzymes, thus allowing the detection of eNOS and proteins bound to eNOS using specific antibodies [14]. Total penile homogenates (30–50 µg) were used for measurements of P-AKT, 4-hydroxy-2-nonenal (HNE) and gp91^{phox} subunit of NADPH oxidase [14,18]. Partially purified NOS samples or penile homogenates were resolved on 4–20% Tris gels and transferred to polyvinylidene difluoride membrane. Membranes with partially purified NOS were probed with anti-phospho (P)-eNOS (Ser-1177) antibody (polyclonal rabbit, Cell Signaling Technology, Beverly, MA) at 1:450 dilution (for P-eNOS analyses) or anti-HSP90 antibody (monoclonal mouse, BD Transduction Laboratories, San Diego, CA) at 1:6,000 dilution (for HSP90 bound to eNOS analysis). After probing for P-eNOS or HSP90, these membranes were stripped and probed with anti-eNOS antibody (monoclonal mouse, BD Transduction Laboratories) at 1:1,000 dilution. P-eNOS and HSP90 densities were normalized relative to that of eNOS. Membranes with penile homogenates were probed with anti-P-AKT (Ser-473) antibody (polyclonal rabbit, Cell Signaling Technology, 1:1,000

dilution), anti-4-HNE antibody at 1:4,000 dilution (polyclonal rabbit, Alpha Diagnostic International, San Antonio, TX, USA), or anti-gp91^{phox} antibody at 1:1,000 dilution (monoclonal mouse, BD Transduction Laboratories, San Diego, CA, USA). Signals were standardized to total AKT (polyclonal rabbit antibody, Cell Signaling Technology, 1:1,000 dilution), or β -actin (monoclonal mouse antibody, Sigma Chemical, St. Louis, MO, USA; 1 : 7,000 dilution). Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, Piscataway, NJ, USA), and quantified using NIH Image 1.29. All results were expressed relative to WT mice treated with vehicle.

Statistical Analysis

Statistical analysis was performed by using oneway analysis of variance, followed by Newman–Keuls multiple comparison test (between treatment groups) or by *t*-test (between control and each treatment group). The data were expressed as the mean $\pm$ standard error of the mean (SEM). A value of $P < 0.05$ was considered to be statistically significant.

Results

Normalization of eNOS Posttranslational Modification in the Sickie Mouse Penis by Continuous Sildenafil Treatment

eNOS phosphorylation on Ser-1177, a positive regulatory site on eNOS, was significantly ($P < 0.05$) reduced in the penis of Sickie compared with WT mice. Continuous treatment of Sickie mice with sildenafil significantly ($P < 0.05$) increased P-eNOS (Ser-1177) to levels similar to the levels found in WT mice (Figure 1A). Similarly, eNOS interaction with HSP90, which also activates eNOS, was significantly ($P < 0.05$) reduced in the penis of Sickie compared with WT mice, and normalized ($P < 0.05$) by continuous treatment with sildenafil (Figure 1B). Sildenafil did not affect P-eNOS (Ser-1177) levels or eNOS interaction with HSP90 in the penis of WT mice.

Normalization of P-AKT Signaling in the Sickie Mouse Penis by Continuous Sildenafil Treatment

Phosphorylation of AKT at Ser-473 is important for its full activation. We next examined whether the stimulatory effect of continuous sildenafil treatment on eNOS phosphorylation at Ser-1177 in the Sickie mouse penis is mediated by P-AKT. The levels of P-AKT were significantly ($P < 0.05$) reduced in the penis of Sickie mice relative to levels in WT mice, and were normalized ($P < 0.05$) after continuous sildenafil treatment. Sildenafil did not affect P-AKT levels in the penis of WT mice (Figure 2).

Normalization of Upregulated Protein Expression of NADPH Oxidase Subunit gp91^{phox} and Oxidative Stress in the Sickie Mouse Penis by Continuous Sildenafil Treatment

Protein expression of NADPH oxidase catalytic subunit gp91^{phox} was significantly ($P < 0.05$) increased in the penis of Sickie compared with WT mice. Continuous treatment of Sickie mice with sildenafil significantly ($P < 0.05$) reduced protein expression of gp91^{phox} (Figure 3). 4-HNE is a major product of lipid peroxidation of polyunsaturated fatty acids, which covalently binds to proteins, causing their malfunction [18]. 4-HNE mimicked changes in NADPH oxidase subunit; it was significantly ($P < 0.05$) upregulated in the penis

of Sickie compared with WT mice, and was significantly ($P < 0.05$) decreased by continuous sildenafil treatment. Sildenafil did not affect gp91^{phox} or 4-HNE protein expressions in the penis of WT mice (Figures 3 and 4).

Discussion

The present study demonstrated that continuous treatment of sickle cell mice with sildenafil promotes eNOS posttranslational activation and inhibits NADPH oxidase and associated oxidative stress in the penis. The promoted eNOS post-translational activation is evidenced by increased interaction of eNOS with HSP90 and increased eNOS phosphorylation on Ser-1177. Decreased oxidative stress is suggested by downregulation of NADPH oxidase catalytic subunit gp91^{phox}.

SCD is characterized by impaired constitutive NOS activity [5,14,16] and eNOS posttranslational activation (decreased phosphorylation at Ser-1177 and decreased interaction with HSP90 (ref. [14] and present data) in the penis. We now show that continuous sildenafil treatment normalizes deleterious effects of SCD on eNOS interaction with HSP90 and eNOS phosphorylation on Ser-1177 in the penis. Our findings are in agreement with previous studies demonstrating beneficial vascular effects of chronic sildenafil treatment on P-AKT and P-eNOS (Ser-1177) in the rat heart exposed to chronic hypoxia [28] and in the penis of aged rats [29]. Although the exact mechanism of sildenafil's action on eNOS in the penis of sickle cell mice is not known, it is conceivably due to increased cGMP levels, augmented blood flow, and enhanced shear stress. Shear stress increases eNOS/HSP90 interaction, promoting the recruitment of AKT to the complex, AKT activation, and eNOS phosphorylation on Ser-1177 [30]. Recently, integrin-linked kinase, one of the sensors of shear stress on endothelial cells, has been implicated in stabilization of eNOS interaction with HSP90 [31]. Further studies are needed to determine whether integrin mediates this action of sildenafil in the SCD penis. Another mechanism of sildenafil action may involve a direct effect of activated protein kinase G on eNOS phosphorylation [32]. Sildenafil did not affect eNOS phosphorylation on Ser-1177 and eNOS interaction with HSP90 in the healthy penis, which is consistent with human and animal studies showing that impaired endothelial function is more predisposed to improvement by sildenafil than is intact endothelial function [16,33].

In addition to its vasculoprotective effect on eNOS function, long-term sildenafil treatment reduces oxidative stress, providing another mechanism to increase NO bioavailability. Chronic sildenafil treatment reduces systemic oxidative stress in insulin resistant rats [34,35], and local oxidative stress in the aorta [36] and heart [37] of diabetic rats and kidney of hypertensive rats [38]. In the aorta of diabetic rats [39,40] this effect of sildenafil has been attributed to the reduction of enhanced protein expression of NADPH oxidase subunits gp91^{phox} and p67^{phox}. Chronic sildenafil treatment also has a beneficial effect on reducing oxidative stress in the penis of aged [41], diabetic [42], and secondhand smoke-induced ED animal models [26], but the mechanism of this action of sildenafil is not known.

SCD is associated with increased oxidative stress in the penis generated by NADPH oxidase, xanthine oxidase, and eNOS uncoupling [15,16,18]. We recently demonstrated that

continuous sildenafil treatment reduces oxidative stress and prevents eNOS uncoupling in the penis of sickle cell mice [16]. Our present study shows that another source of ROS in the penis of sickle mice is also a target of sildenafil, as continuous treatment with sildenafil decreased upregulated NADPH oxidase catalytic subunit gp91^{phox} and oxidative stress. This effect of sildenafil action on NADPH oxidase may be due to the inhibitory action of the NO/cGMP on NADPH oxidase expression. Indeed, in human endothelial cells NO inhibits NADPH oxidase activity via direct S-nitrosylation of its regulatory p47^{phox} and catalytic NOX5 subunits [43,44]. In human umbilical vein endothelial cells [45] laminar shear stress inhibits protein expression of NADPH oxidase subunits gp91^{phox} and p47^{phox}. These findings indicate that the beneficial vascular effects of continuous sildenafil treatment in the penis in association with SCD involve improvement of eNOS function by post-translational activation and inhibition of superoxide-producing enzymes NADPH oxidase and eNOS uncoupling.

The novelty of our findings herein refers specifically to the beneficial effect of continuous sildenafil treatment on the SCD penis via: (i) promoting eNOS interaction with HSP90 and eNOS phosphorylation on Ser-1177 in the penis (eNOS posttranslational activation); (ii) decreasing upregulated NADPH oxidase catalytic subunit gp91^{phox} (NADPH oxidase-associated oxidative stress).

A limitation of our study is the lack of erectile function measurement in Sickle mice to directly correlate physiologic and molecular findings in the same animal. However, we performed molecular studies at a time point when animals have previously been reported to exhibit priapism [16,19,21]. We believe that the molecular benefits observed through sildenafil treatment in an animal model for SCD may therefore represent a more general effect on corporal/vascular tissue which would be beneficial for correcting priapism associated with SCD.

Conclusion

In conclusion, continuous treatment with PDE5 inhibitor sildenafil restores eNOS interaction with HSP90, increases AKT-mediated phosphorylation of eNOS on Ser-1177, and prevents oxidative stress generated by NADPH oxidase in the penis in association with SCD. These data indicate a multifactorial basis for possible beneficial effects of sildenafil in improving erectile function affected by priapism.

Acknowledgments

This work was supported by US Public Service Grants HL090515 and RO1DK067223 (to ALB).

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Figure 1.

Effect of continuous sildenafil treatment on (A) P-eNOS (Ser-1177) and (B) eNOS/HSP90 interaction in penes. WT and Sickle mice were treated with sildenafil or vehicle for 3 weeks. Upper panels are representative Western immunoblots of P-eNOS (Ser-1177)/eNOS and HSP90/eNOS in penes of WT, WT treated with sildenafil, Sickle, and Sickle mice treated with sildenafil. Lower panel represents quantitative analysis of P-eNOS (Ser-1177)/ eNOS and HSP90/eNOS in penes in the same treatment groups. Each bar represents the mean $\pm$ SEM of 5–7 mice. * $P < 0.05$ vs. WT; ** $P < 0.05$ vs. Sickle.

Figure 2.

Effect of continuous sildenafil treatment on P-AKT in penes. Upper panels are representative Western immunoblots of P-AKT/AKT in penes of WT, WT treated with sildenafil, Sickle, and Sickle mice treated with sildenafil. Lower panel represents quantitative analysis of P-AKT/AKT in penes in the same treatment groups. Each bar represents the mean $\pm$ SEM of 5 mice. * P < 0.05 vs. WT; ** P < 0.05 vs. Sickle.

Figure 3.

Effect of continuous sildenafil treatment on NADPH oxidase catalytic subunit gp91^{phox} in penes. Upper panels are representative Western immunoblots of gp91^{phox}/ β -actin in penes of WT, WT treated with sildenafil, Sickle, and Sickle mice treated with sildenafil. Lower panel represents quantitative analysis of gp91^{phox}/ β -actin in penes in the same treatment groups.

Each bar represents the mean $\pm$ SEM of 4–6 mice. * P < 0.05 vs. WT; ** P < 0.05 vs. Sickle.

Figure 4.

Effect of continuous sildenafil treatment on 4-HNE in penes. Upper panels are representative Western immunoblots of 4-HNE/β-actin in penes of WT, WT treated with sildenafil, Sickle, and Sickle mice treated with sildenafil. Lower panel represents quantitative analysis of 4-HNE/β-actin in penes in the same treatment groups. The analysis of 4-HNE is a densitometric composite of all proteins in each lane. Each bar represents the mean $\pm$ SEM of 5–6 mice. * $P < 0.05$ vs. WT; ** $P < 0.05$ vs. Sickle.