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Posttranslational inactivation of endothelial nitric oxide synthase in the transgenic sickle cell mouse penis

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Abstract

INTRODUCTION—Sickle cell disease (SCD)-associated priapism is characterized by endothelial nitric oxide synthase (eNOS) dysfunction in the penis. However, the mechanism of decreased eNOS function/activation in the penis in association with SCD is not known.

AIMS—Our hypothesis in the present study was that eNOS is functionally inactivated in the SCD penis in association with impairments in eNOS posttranslational phosphorylation and the enzyme's interactions with its regulatory proteins.

METHODS—Sickle cell transgenic (sickle) mice were used as an animal model of SCD. Wild type (WT) mice served as controls. Penes were excised at baseline for molecular studies. eNOS phosphorylation on Ser-1177 (positive regulatory site) and Thr-495 (negative regulatory site), total eNOS, and phosphorylated AKT (upstream mediator of eNOS phosphorylation on Ser-1177) expressions, and eNOS interactions with heat shock protein 90 (HSP90) and caveolin-1 were measured by Western blot. Constitutive NOS catalytic activity was measured by conversion of L-[¹⁴C]arginine-to-L-[¹⁴C]citrulline in the presence of calcium.

MAIN OUTCOME MEASURES—Molecular mechanisms of eNOS dysfunction in the sickle mouse penis.

RESULTS—eNOS phosphorylated on Ser-1177, an active portion of eNOS, was decreased in the sickle mouse penis compared to WT penis. eNOS interaction with its positive protein regulator HSP90, but not with its negative protein regulator caveolin-1, and phosphorylated AKT expression, as well as constitutive NOS activity, were also decreased in the sickle mouse penis compared to WT penis. eNOS phosphorylated on Thr-495, total eNOS, HSP90, and caveolin-1 protein expressions in the penis were not affected by SCD.

CONCLUSION—These findings provide a molecular basis for chronically reduced eNOS function in the penis by SCD, which involves decreased eNOS phosphorylation on Ser-1177 and decreased eNOS-HSP90 interaction.

Keywords

Priapism; eNOS phosphorylation; HSP90; caveolin-1

INTRODUCTION

Sickle cell disease (SCD) is a hemoglobinopathy resulting from the expression of abnormal sickle hemoglobin (HbS). HbS arises from a single point mutation that substitutes amino acid valine for glutamic acid in the β -globin subunit of hemoglobin, leading to aggregation of sickle hemoglobin and red blood cell rigidity; abnormal red blood cells are associated with poor blood flow resulting in tissue hypoxia and ischemia.¹ In addition to hemoglobinopathy and red blood cell sickling, SCD features an independent spectrum of vascular dysfunction, involving such abnormalities as defects in nitric oxide (NO) bioavailability, enhanced responses to vasoconstrictors, and elevated oxidative stress.² The most common vasculopathies of SCD are pulmonary hypertension, priapism, and stroke.

Priapism is an erection disorder consisting of non-willful, excessive, and often recurrent penile erection unrelated to sexual excitement. It afflicts about 40% of men with SCD.³ Ischemic priapism, the most common form of priapism in which blood flow in the corpora cavernosa is absent, is frequently associated with irreversible penile tissue necrosis, as well as permanent and irreversible erectile dysfunction.⁴ Priapism has been shown to be associated with the overproduction of adenosine in the penis and activation of A2B receptor.^{5, 6} Furthermore, recent scientific investigation in animals has demonstrated that priapism arises from chronically impaired NO bioavailability due to a defect in the NO/cGMP/phosphodiesterase (PDE)5 signaling pathway in the penis.^{7, 8} Calcium-dependent NO synthase (NOS) activity and cGMP production are basally downregulated in the penis of endothelial NOS (eNOS) knockout mice, combined eNOS and neuronal NOS knockout mice, and sickle cell transgenic (sickle) mice, which downregulates PDE5 in the penis of these mice.⁷ Upon neuronally stimulated penile erection, priapism occurs due to uncontrolled cGMP-dependent vasodilation of the corpora cavernosa resulting from downregulated PDE5.^{7, 9, 10} These findings point to the critical role of eNOS dysfunction in the penis as a major molecular disturbance associated with priapism. However, the mechanism of decreased eNOS function or activation in the penis in association with SCD is not known.

AIMS

Our hypothesis in the present study was that eNOS is functionally inactivated in the SCD penis in association with SCD. Correlatively, we hypothesized that eNOS activation mechanisms are impaired by posttranslational modifications of the enzyme, such as its phosphorylation and its interactions with regulatory proteins, heat shock protein 90 (HSP90)

and caveolin-1. For this investigation, we used the sickle transgenic mouse as an animal model of SCD.

MATERIALS AND METHODS

Mouse model of human sickle cell disease

Transgenic sickle mice with knockout of all mouse hemoglobin genes and expressing exclusively human sickle hemoglobin (HbS) were developed at Lawrence Berkeley National Laboratory.¹¹ An in-house breeding colony, which mated sickle male mice to hemizygous females, generated animals for this study. Genotyping was performed by Transnetyx, Inc. (Cordova, TN). These 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.^{12, 13} Aged-matched WT C57BL/6 males were used as controls because this represents the predominant background strain for the transgenic mice. Male mice were 7–9 months old. Mice were pathogen free and received routine NIH rodent chow and water. Flaccid penes were collected before WT (n=13) and sickle (n=13) 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.¹⁴ NOS was partially purified by affinity binding to 2',5'-ADP Sepharose, an NADP structural analog immobilized on Sepharose 4B, which binds and immobilizes enzymes requiring this cofactor.¹⁴ Partially purified NOS samples 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 and Thr-495) antibodies (polyclonal rabbit, Cell Signaling Technology, Beverly, MA) at 1:450 dilution (for P-eNOS analyses), anti-caveolin-1 antibody (polyclonal rabbit, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution (for caveolin-1 bound to eNOS analysis), or anti-HSP90 antibody (monoclonal mouse, BD Transduction Laboratories, San Diego, CA) at 1:6,000 dilution (for HSP90 bound to eNOS analysis).^{15–17} After probing for P-eNOS, caveolin-1, or HSP90, these membranes were stripped and probed with anti-eNOS antibody (monoclonal mouse, BD Transduction Laboratories) at 1:1,000 dilution. P-eNOS, caveolin-1, and HSP90 densities were normalized relative to those of eNOS in partially purified samples. For Western blot analysis of P-AKT (Ser-473, polyclonal rabbit antibody, Cell Signaling Technology, 1:1,000 dilution), total caveolin-1 (1:7,000 dilution), total eNOS (1:1,000 dilution), and total HSP90 (1:2,000 dilution), a separate set of homogenates (20–70 µg) was used without purification and standardized to AKT (polyclonal rabbit antibody, Cell Signaling Technology, 1:1,000 dilution), or β -actin (monoclonal mouse antibody, Sigma Chemical, St. Louis, MO, 1:7,000 dilution). The ratio was determined in terms of arbitrary units and expressed relative to the ratio for WT mice. To verify that β -actin expression was not affected by SCD, the density of a set of samples was standardized per total proteins (Ponceau S staining). Ratios did not differ from the ones obtained using β -actin for standardization (data not shown). Band densities were quantified using NIH Image 1.29.

Constitutive NOS Enzyme Assay

Penile constitutive NOS catalytic activity was assayed by L-[¹⁴C]arginine-to-L-[¹⁴C]citrulline conversion in the presence of calcium.¹⁸ Enzyme activity was expressed as L-citrulline production in counts/min.

Statistical evaluation

Statistical analysis was performed by using the t-test for testing hypotheses for significant differences in mean values between groups. The data were expressed as the mean ± standard error of the mean (SEM). A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

P-eNOS (Ser-1177 and Thr-495) and eNOS expressions in the penis of sickle mice

Because eNOS phosphorylated at Ser-1177 is the main activated form of the enzyme, we assessed the amount of P-eNOS (Ser-1177) in the penis of sickle mice. The sickle mouse penis exhibited significantly ($P < 0.05$) reduced expression of P-eNOS (Ser-1177) compared to that of WT mice (Figure 1A). In contrast to eNOS phosphorylation on Ser-1177, phosphorylation of eNOS on Thr-495, which decreases the enzyme's activity, was not affected by SCD (Figure 1B). Total eNOS protein expression in the penis of sickle mice did not differ from that of WT mice (Figure 1C).

P-AKT in the penis of sickle mice

P-AKT, an upstream mediator of eNOS phosphorylation on Ser-1177, was significantly ($P < 0.05$) reduced in the penis of sickle mice compared to the WT mouse penis (Figure 2).

eNOS bindings to HSP90 and caveolin-1 in the penis of sickle mice

eNOS phosphorylation on Ser-1177 and eNOS activity are in large part determined by eNOS interaction with its main positive regulatory protein HSP90 and negative regulatory protein caveolin-1. Bindings of eNOS to HSP90 and caveolin-1 were evaluated in the penile samples partially purified for NOSs, thus allowing the detection of eNOS and proteins bound to eNOS using specific antibodies.^{15–17} While coimmunoprecipitation of eNOS and HSP90 would further support their interactions in the penis, these types of experiments in tissues are technically difficult, in contrast to using isolated endothelial cells. Figure 3A shows that the ratio of HSP90/eNOS was significantly ($P < 0.05$) decreased in the penis of sickle mice compared to that of WT mice, implying decreased eNOS interaction with HSP90. These changes were unrelated to HSP90 expression, which was not different in the penis of sickle mice compared to that of WT mice (Figure 3B). eNOS binding to its negative regulator caveolin-1 (Figure 4A), as well as the protein expression of caveolin-1 (Figure 4B) in the penis of sickle mice, did not differ from values in WT mice.

Constitutive NOS activity in the penis of sickle mice

Conversion of L-arginine to L-citrulline in the presence of calcium, a direct measure of constitutive (eNOS and neuronal NOS) catalytic activity, was decreased in penes from sickle

mice (1463.8 ± 227.3 counts/min; $n=5$) compared to WT mouse penis (3885.2 ± 528.2 counts/min; $n=5$) at baseline.

DISCUSSION

The results of the present study demonstrate that altered eNOS regulatory mechanisms occur in the SCD penis, affirming that this condition features functionally inactivated eNOS. Here we show a decrease in eNOS phosphorylation on Ser-1177, an active portion of eNOS, in the SCD mouse penis. This effect was apparently due to decreased eNOS interaction with its positive protein regulator HSP90 (but not increased interaction with caveolin-1), which decreases AKT activation and AKT-mediated eNOS phosphorylation on Ser-1177, and reduces constitutive NOS activity. These findings provide a molecular basis for chronically reduced NO bioavailability in the penis by SCD, which conceivably results in priapism upon neuronally stimulated penile erection.⁸ These data also identify a new molecular target potentially for pharmacologic therapies for SCD, although more mechanistic studies will be required to demonstrate how eNOS/HSP90 interaction can be regulated or adjusted.

eNOS catalyzes the synthesis of NO, a potent vasodilator and a key mediator of penile erection and vascular homeostasis.¹⁹ The bioavailability of endothelial NO is largely regulated by posttranslational regulation of eNOS, such as binding of calcium-calmodulin, phosphorylation, protein interaction, dimer stabilization, and localization.²⁰ While activation of eNOS with calcium/calmodulin is essential to the first step of the catalytic process of NO production, phosphorylation of eNOS at Ser-1177 is a key posttranslational modification that increases electron transfer at low intracellular calcium levels, which ensures the constitutive production of NO.²⁰ Phosphorylation of eNOS at this site correlates directly with electron flow through the eNOS enzyme, increases in eNOS activity, and NO production.²¹ The decrease in eNOS phosphorylation on Ser-1177 in the penis of SCD mice, observed in this study, thus refers to a decrease in the active portion of the enzyme. This is evidenced by decreased calcium-dependent NOS activity in the SCD mouse penis, which conceivably reduces endothelial NO bioavailability and results in decreased basal blood flow and vascular dysfunction in the SCD mouse penis.

We conducted further studies to determine the mechanism of decreased eNOS phosphorylation on Ser-1177 in the penis of SCD mice. The transition from the early calcium-dependent to the phosphorylation-dependent activation of eNOS requires binding of eNOS to its main positive regulatory protein HSP90.²² HSP90 is associated with eNOS in the resting state, and upon stimulation of endothelial cells with agonists such as VEGF and shear stress, the association between the two proteins is increased, concomitant with enhanced NO production.²³ The mechanism of eNOS activation upon HSP90 binding involves calmodulin-dependent disruption of eNOS binding with caveolin-1, recruitment of eNOS and AKT to adjacent regions on HSP90, reduced dephosphorylation of AKT, and increased ability of AKT to phosphorylate HSP90-bound eNOS.^{23–27} The direct protein interaction between eNOS and HSP90 also enhances eNOS coupled activity and prevents superoxide production by eNOS.^{28–30} Decreased eNOS-HSP90 interaction has been demonstrated in the lungs of sickle mice compared to WT mice.³¹ This effect has been attributed to increased oxidative stress generated by activated xanthine oxidase. We now

show decreased binding of eNOS and HSP90 in the penis of SCD mice, which conceivably prevents AKT activation by phosphorylation and AKT-mediated eNOS phosphorylation on Ser-1177, and thus prevents eNOS activation. The decrease in HSP90 interaction with eNOS was not due to lower HSP90 or eNOS protein levels, which were not affected by SCD. Further studies are needed to determine the mechanism underlying the decreased interaction between HSP90 and eNOS in the SCD mouse penis. In addition to oxidative stress which may decrease eNOS-HSP90 interaction, the carboxyl terminus of HSP70-interacting protein (CHIP) has been shown to inactivate eNOS by uncoupling its interaction with HSP90 and by displacement of eNOS from the Golgi apparatus, which is otherwise required for trafficking of eNOS to the plasmalemma and subsequent activation.³² Furthermore, the function of HSP90 may be affected by its interaction with stimulatory and inhibitory proteins, phosphorylation, acetylation, and *S*-nitrosylation, the latter affecting the HSP90 conformation and binding to eNOS.^{33, 34}

In contrast to eNOS phosphorylation on Ser-1177 which activates eNOS, eNOS phosphorylation on Thr-495 reduces eNOS catalytic activity by interfering with binding of calcium/calmodulin to eNOS.^{35–37} Agonist-induced dephosphorylation of Thr-495, associated with increased eNOS activity, is mediated by Ser/Thr protein phosphatase 1 (PP1), Ser/Thr protein phosphatase 2A (PP2A), and calcineurin (PP2B).^{25, 36, 38} In vitro studies demonstrated that the activity of calcineurin is increased by HSP90.³⁹ In human umbilical vein endothelial cells HSP90 overexpression promotes its association with eNOS, AKT, and calcineurin, and stimulates not only eNOS (Ser-1177) phosphorylation, but also eNOS (Thr-495) dephosphorylation, which both activate eNOS.²⁵ We found that SCD did not affect eNOS phosphorylation on Thr-495 in the mouse penis, implying that decreased eNOS interaction with HSP90 in the sickle mouse penis specifically affects Ser-1177 phosphorylation and not dephosphorylation of eNOS on Thr-495.

While HSP90 facilitates eNOS activation, caveolin-1 operates as a main negative regulator of eNOS.⁴⁰ The majority of functional eNOS in quiescent endothelial cells is targeted to caveolae by cotranslational N-myristoylation and posttranslational palmitoylation.²⁰ Caveolin-1, the resident membrane protein of caveolae, can directly interact with eNOS and inhibit its activity by occupying its calmodulin-binding site.^{41–43} Stimuli such as shear stress and estrogen induce calcium increase, and the calcium/calmodulin complex displaces eNOS from caveolin-1 and away from its tonic inhibition. eNOS translocation is associated with interaction with other proteins, such as calmodulin and HSP90, and eNOS activation.⁴⁴ We recently showed that increased interaction between eNOS and caveolin-1 occurs in the hypercholesterolemic pig penis,¹⁶ which apparently prevented eNOS activation and contributed to endothelial dysfunction. The interaction between eNOS and HSP90 was unaffected in the pig penis (unpublished data), implying specifically that the eNOS-caveolin-1 interaction in the penis is decreased in association with hypercholesterolemia. The present study demonstrates that eNOS interaction with caveolin-1 in the SCD mouse penis was unaffected. Our findings imply that dysregulated eNOS interaction with HSP90 associated with decreased phosphorylation of eNOS on Ser-1177 and decreased constitutive NOS activity may be a major mechanism underlying basally reduced eNOS function in the SCD mouse penis. Further studies are needed to examine whether other eNOS regulatory mechanisms, such as increased oxidative stress, changes in phosphorylation on other sites on

eNOS, phosphorylation mediated by kinases other than AKT, phosphatase action, or interaction of eNOS with other regulatory proteins, are contributory.

At first it seems counterintuitive that decreased basal eNOS function leads to priapism. Recent studies demonstrated that the priapic behavior can be explained by chronically reduced endothelial NO bioavailability, resulting in PDE5 downregulation. As a result of PDE5 downregulation, cGMP accumulates in the penis after an episode of neurostimulation or nocturnal erection, producing unrestrained erectile tissue relaxation.^{7–10, 45}

In addition to impaired endothelial NO signaling, as shown in this study, RhoA/ROCK-mediated vasoconstriction is decreased in penes of SCD mice, contributing to increased vasorelaxation.¹³

CONCLUSION

We observed altered eNOS phosphorylation on Ser-1177, a posttranslational modification of the enzyme which is a key regulator of its activity, in the SCD mouse penis. The underlying mechanism involves decreased eNOS interaction with its positive protein regulator HSP90, which decreases AKT activation and AKT-mediated eNOS phosphorylation on this site, decreasing constitutive NOS catalytic activity.

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Figure 1.

Effect of SCD on P-eNOS (Ser-1177) (A), P-eNOS (Thr-495) (B) and total eNOS (C) expressions in the penis of WT and sickle mice at baseline. Upper panels are representative Western immunoblots. Lower panels represent quantitative analyses of P-eNOS (Ser-1177), P-eNOS (Thr-495), and total eNOS in penes of WT and sickle mice. Each bar represents the mean $\pm$ SEM. *, $P < 0.05$. $n = 6-8$

Figure 2. Effect of SCD on P-AKT in the penis of WT and sickle mice at baseline. Upper panel is a representative Western immunoblot, and lower panel represents a quantitative analysis of P-AKT in penes of WT and sickle mice. Each bar represents the mean $\pm$ SEM. *, $P < 0.05$. $n=8$

Figure 3.

Effect of SCD on HSP90 binding to eNOS (A) and total HSP90 protein expression (B) in the penis of WT and sickle mice at baseline. Upper panels are representative Western blots, and lower panels represent quantitative analyses of HSP90/eNOS (indicative of HSP90 binding to eNOS) and HSP90 protein expression in penes of WT and sickle mice. Each bar represents the mean $\pm$ SEM. *, $P < 0.05$. $n=5$

Figure 4. Effect of SCD on caveolin-1 binding to eNOS (A) and total caveolin-1 protein expression (B) in the penis of WT and sickle mice at baseline. Upper panels are representative Western blots, and lower panels represent quantitative analyses of caveolin-1/eNOS (indicative of caveolin-1 binding to eNOS) and caveolin-1 protein expression in penes of WT and sickle mice. Each bar represents the mean $\pm$ SEM. $n=5-8$

Figure 5.

Model for altered eNOS regulatory mechanisms in the SCD mouse penis. eNOS phosphorylation on Ser-1177, an active portion of eNOS, is decreased in the SCD mouse penis. This effect is due to decreased eNOS interaction with its positive protein regulator HSP90 (but not increased interaction with caveolin-1), which decreases AKT activation and AKT-mediated eNOS phosphorylation on Ser-1177, and reduces constitutive NOS activity. Decreased eNOS interaction with HSP90 in the sickle mouse penis does not affect dephosphorylation of eNOS on Thr-495.