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Transnitrosylation: A Factor in Nitric Oxide—Mediated Penile Erection

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ABSTRACT

Introduction: Nitric oxide (NO) signaling can be mediated not only through classic 3′,5′-cyclic guanosine monophosphate but also through *S*-nitrosylation. However, the impact of *S*-nitrosylation on erectile function and in NO regulation and oxidative stress in the penis remains poorly understood.

Aims: To characterize the role of *S*-nitrosoglutathione reductase (GSNOR), a major regulator of *S*-nitrosylation homeostasis, on erection physiology and on endothelial NO synthase (eNOS) function and oxidative-nitrosative stress in the penis.

Methods: Adult GSNOR-deficient and wild-type (WT) mice were used. Erectile function was assessed in response to electrical stimulation of the cavernous nerve. Total NO in penile homogenates was measured by Griess reaction. Protein *S*-nitrosylation, eNOS phosphorylation on Ser-1177 (positive regulatory site), eNOS uncoupling, and markers of oxidative stress (4-hydroxy-2-nonenal, malondialdehyde, and nitrotyrosine) in the penis were measured by western blot.

Main Outcome Measures: Erectile function, eNOS function, and oxidative stress in the penis of GSNOR-deficient mice.

Results: Erectile function was intact in GSNOR-deficient mice. Total *S*-nitrosylated proteins were increased ($P < .05$) in the GSNOR$^{-/-}$ compared with WT mouse penis. Although eNOS phosphorylation on Ser-1177 did not differ between the GSNOR$^{-/-}$ and WT mouse penises at baseline, electrical stimulation of the cavernous nerve increased ($P < .05$) phosphorylated eNOS in the WT mouse penis but failed to increase phosphorylated eNOS in the GSNOR$^{-/-}$ mouse penis. Total NO production was decreased ($P < .05$), whereas eNOS uncoupling, 4-hydroxy-2-nonenal, malondialdehyde, and nitrotyrosine were increased ($P < .05$) in the GSNOR-deficient mouse penis compared with the WT mouse penis.

Conclusion: Transnitrosylation mechanisms play an important role in regulating NO bioactivity in the penis. Deficiency of GSNOR leads to eNOS dysfunction and increased oxidative damage, suggesting that homeostatic eNOS function in the penis is governed by transnitrosylation.

Key Words: *S*-Nitrosylation; *S*-Nitrosoglutathione Reductase; Mouse; Endothelial Nitric Oxide Synthase; Endothelial Nitric Oxide Synthase Uncoupling; Oxidative-Nitrosative Stress; Erectile Function

INTRODUCTION

Penile erection is a complex process involving neurogenic, psychogenic, and hormonal mechanisms, with nitric oxide (NO) generally accepted as the main mediator.[1] Traditional views of NO signaling in the penis suggest that NO is released by endothelium and neurons in penile tissue and binds to the heme group of soluble guanylyl cyclase to increase the production of 3′,5′-cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G. cGMP-protein kinase G regulates intracellular signaling, which causes the relaxation of smooth muscle in the corpora cavernosa and penile erection.[1]

Recent evidence has shown that many actions of NO can be mediated independently of cGMP signaling and are controlled enzymatically through *S*-nitrosylation. *S*-nitrosylation is a non-enzymatic reversible reaction consisting of the covalent attachment of an NO moiety to a reactive cysteine residue to form *S*-nitrosothiols (SNOs), which include *S*-nitrosylated proteins and low-molecular-weight *S*-nitrosoglutathione (GSNO). In addition, *S*-nitrosylation can occur through transnitrosylation involving GSNO and an acceptor thiol.[2,3] GSNO, the most abundant endogenous SNO, serves as a stable intracellular reservoir of NO. Immunologic detection of SNOs suggests that

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the NO synthase (NOS) isoforms, endothelial NOS (eNOS), neuronal NOS, and inducible NOS, can be included among *S*-nitrosylated proteins existing in diverse tissues ranging from endothelium to developing neurons and throughout blood vessel walls.[4]

A key mechanism for regulating the action of SNOs involves the enzyme alcohol dehydrogenase III, also known as *S*-nitrosoglutathione reductase (GSNOR).[5,6] GSNOR selectively metabolizes GSNO and thereby indirectly depletes the levels of *S*-nitrosylated proteins, which are in a dynamic equilibrium with GSNO.[5] GSNOR deficiency results in markedly increased levels of GSNO and *S*-nitrosylated proteins.[7,8] Thus, changes in GSNOR activity affect the entire SNO pool and thus might modulate cellular signaling.[5,6]

We previously reported GSNOR localizations in penile nerves and in vascular endothelium and smooth muscle cells of penile blood vessels.[9] However, the significance of transnitrosylation in erection physiology and in NO regulation and oxidative stress in the penis requires elucidation. In this study, we used GSNOR-deficient mice to characterize the role of GSNOR in erectile function and in eNOS function and oxidative-nitrosative stress in the penis.

METHODS

Animals

Adult male (3- to 5-month-old) homozygous GSNOR-deficient ($GSNOR^{-/-}$) and age-matched wild-type (WT) mice (C57BL/6, The Jackson Laboratory, Bar Harbor, ME, USA) were used. Animals were cared for and housed under strict guidelines and all procedures were approved by The Johns Hopkins University School of Medicine animal care and use committee (Baltimore, MD, USA).

In Vivo Erection Studies

Mice were anesthetized with ketamine 100 mg/kg plus xylazine 10 mg/kg by intraperitoneal injection. To monitor mean arterial pressure (MAP), the right carotid artery was cannulated with polyethylene tubing filled with heparinized saline (100 U/mL). To monitor changes in intracavernosal pressure (ICP), the penis was denuded of skin and fascia and a 30-gauge needle connected by polyethylene tubing to a pressure transducer was inserted into the right crus. The cavernous nerve was affixed with a bipolar electrode attached to a Grass Instruments S48 stimulator (Quincy, MA, USA) and stimulated at 1, 2, and 4 V at 16 Hz with a 5-ms square-wave duration for 1 minute.[10] ICP was recorded using the DI-190 system (Dataq Instruments, Akron, OH, USA) from the start of electrical stimulation until 60 seconds after stimulation ended. Erectile function was represented by the normalized maximal ICP/MAP and the ratio of total area under the curve to MAP (total ICP). Results were analyzed using MATLAB (Mathworks, Natick, MA, USA).

NO Assay

Griess reaction measuring the total amount of nitrite and nitrate was performed with a commercially available kit (Oxford Biomedical Research, Rochester Hills, MI, USA) on penile tissue collected at baseline, as previously described.[11] This kit uses metallic cadmium for quantitative conversion of nitrate to nitrite before quantitation of nitrite using the Griess assay, thus providing for accurate determination of total NO (NO_x) production. For Griess assays, absorbance was measured at 540 nm using a 680-nm microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blot Analysis

For measurements of basal and stimulated levels of phosphorylated (P)-eNOS (Ser-1177), penises were collected at baseline and 1 minute after a single electrical stimulation of the cavernous nerve at 4 V for 60 seconds, respectively. Penises were immediately snap frozen in liquid nitrogen and homogenized, as described previously.[10] NOS was partly purified and probed with polyclonal rabbit anti—P-eNOS (Ser-1177) antibody (Cell Signaling Technology, Beverly, MA, USA) at 1:450 dilution; then, the membrane was stripped and probed with polyclonal rabbit anti-eNOS antibody (BD Transduction Laboratories, San Diego, CA, USA) at 1:1,000 dilution.[12] In a separate group of mice, penises were removed from animals at baseline for measurements of eNOS uncoupling, protein *S*-nitrosylation, and markers of oxidative stress. For the analysis of dimeric and monomeric forms of eNOS, low-temperature sodium dodecyl sulfate gel electrophoresis was used with partly purified penile homogenates, as described previously[13]; then, membranes were probed with polyclonal rabbit anti-eNOS antibody at 1:1,000 dilution. For analysis of oxidative stress markers and *S*-nitrosylated proteins, membranes were probed with polyclonal rabbit anti—4-hydroxy-2-nonenal (4-HNE) antibody (Alpha Diagnostic International, San Antonio, TX, USA) at 1:5,000 dilution, polyclonal rabbit anti—*S*-nitrocysteine, polyclonal rabbit anti-malondialdehyde (MDA), and monoclonal mouse anti-nitrotyrosine antibodies (Abcam Inc, Cambridge, MA, USA) at 1:500, 1:500, and 1:2,000 dilutions, respectively.[14] Signals were standardized to β-actin (monoclonal mouse antibody at 1:7,000 dilution; Sigma Chemical, St. Louis, MO, USA). Bands were detected by horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (GE Healthcare, Piscataway, NJ, USA) and analyzed using NIH Image software. P-eNOS density was normalized relative to that of eNOS in partly purified samples. eNOS uncoupling was represented inversely as a ratio of active eNOS dimers to inactive eNOS monomers. The analysis of 4-HNE, MDA, nitrotyrosine, and protein-SNO was a densitometric composite of all proteins in each lane. All results were expressed relative to WT data.

Statistical Analyses

Statistical analyses were performed using two-way repeated-measures analysis of variance and Student t-test (SigmaStat