Phosphodiesterase-5A dysregulation in penile erectile tissue is a mechanism of priapism


Departments of *Cardiology and †Urology, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21287

Edited by Louis J. Ignarro, University of California School of Medicine, Los Angeles, CA, and approved December 15, 2004 (received for review September 28, 2004)

The molecular mechanism for priapism is not well characterized. Although the nitric oxide (NO) pathway is known to mediate penile erection under normal conditions, we hypothesized that the mechanism of priapism rests in aberrant downstream signaling of this pathway based on our previous findings that mice lacking the gene for endothelial nitric oxide synthase (eNOS<sup>−/−</sup>) and mice lacking both neuronal NOS (nNOS) and eNOS (nNOS<sup>−/−</sup>, eNOS<sup>−/−</sup>) have a tendency for priapic activity. We investigated the role of downstream guanylate cyclase and phosphodiesterase type 5 (PDE5A) expression and function in mediating these responses in eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup>, eNOS<sup>−/−</sup> mice. Erectile responses to both cavernous nerve stimulation and intracavernosal injection of the NO donor diethylamine-NONOate were augmented in eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup>, eNOS<sup>−/−</sup> mice but not in WT or nNOS<sup>−/−</sup> mice. PDE5A protein expression and activity and cGMP levels were significantly lower in eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup>, eNOS<sup>−/−</sup> mice, and this effect was reproduced in WT corpus cavernosum exposed to NO inhibitors. Moreover, cavernous nerve stimulation was associated with a marked augmentation of cavernosal cGMP levels, suggesting that, although lower at baseline, the production of cGMP is unchanged in eNOS<sup>−/−</sup> and nNOS<sup>−/−</sup>, eNOS<sup>−/−</sup> mice upon neurostimulation. Transfection of eNOS<sup>−/−</sup> mice with an adenosine encoding eNOS resulted in a normalization of PDE5A protein and activity as well as a correction of priapic activity. Coupled with the observation that sickle cell disease mice (which show a priapism phenotype) evinced dysregulated PDE5A expression/activity, these data suggest that PDE5A dysregulation is a fundamental mechanism for priapism.

Physiologic Erection Studies. Mice were anesthetized initially by being placed in a jar containing isoflurane-soaked gauze and then intubated and ventilated in supine position with 95% O<sub>2</sub>/5% CO<sub>2</sub> and 2% isoflurane by using a custom-designed, constant-flow mouse ventilator with tidal volume set to 6.7 μl/g at 140 breaths per min. Carotid artery and jugular vein cannulation were performed for systemic monitoring and administration of drugs. Surgical pelvic dissection was performed for cavernous nerve electrical stimulation and intracavernosal drug administration and for intracavernous pressure (ICP) monitoring (11). For studies involving pharmacologic erectile stimulation, sildenafil citrate (Pfizer, Groton, CT), diethylamine-NONOate (DEA/NO), and calcitonin gene-related peptide (Sigma) were prepared in a volume of 10–15 μl/ml, or AdCMV-CNOS (1 × 10<sup>12</sup> parts/ml), or AdCM-VeNOS (1 × 10<sup>12</sup> parts/ml) was injected into a corpus cavernosum with a 30-gauge needle attached to a microliter syringe (12, 13). Mice were recovered and then studied 3 days later (at a time point that was determined in preliminary studies to result in maximum transgene expression).

In Vivo Gene Transfer. After anesthesia as described above, the penis was exposed by sterile technique, and 10 μl of vehicle (3% sucrose in PBS), AdCMVβgal (1 × 10<sup>12</sup> parts/ml), or AdCM-VeNOS (1 × 10<sup>12</sup> parts/ml) was injected into a corpus cavernosum with a 30-gauge needle attached to a microliter syringe. Ganglia were harvested and frozen sections were made for ICP monitoring (11). For studies involving pharmacologic erectile stimulation, sildenafil citrate (Pfizer, Groton, CT), diethylamine-NONOate (DEA/NO), and calcitonin gene-related peptide (Sigma) were prepared in a volume of 10–15 μl/ml for PBS injection. All agents were prepared on the day of the experiment.

Materials and Methods

Animal Models. Age-matched, adult male nNOS<sup>−/−</sup> (NOS1<sup>−/−</sup>), eNOS<sup>−/−</sup> (NOS3<sup>−/−</sup>), and double mutant nNOS<sup>−/−</sup>, eNOS<sup>−/−</sup> mice (NOS1<sup>−/−</sup>/NOS3<sup>−/−</sup>), and WT littermate mice were used (Supporting Text, which is published as supporting information on the PNAS web site). The NOS mutant mice were originally developed on a B6/129 hybrid background strain and backcrossed >12 generations on a C57BL/6 strain (6–9). Transgenic sickle cell (SS) mice, expressing exclusively human sickle hemoglobin, were also used. These mice were age-matched, adult male homozygotes [SS<sup>−/−</sup>, consisting of a transgene and two targeted gene deletions: (Tg(Hu-minILCR alpha 1 g gamma A gamma delta beta s) Hba0/Hbb0/Hbb0/Hbb0)] and heterozygotes (SS<sup>+/−</sup>, SS<sup>−/−</sup> genotype except Hbb0/Hbb0), developed on a mixed-strain background (mouse strains FVB/N, 129, DBA/2, C57BL/6, Black Swiss) (10).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PDE5A, phosphodiesterase type 5A; NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; SS, sickle cell; ICP, intracavernous pressure; DEA/NO, diethylamine-NONOate.

*To whom correspondence should be addressed. E-mail: aburnett@jhmi.edu.

© 2005 by The National Academy of Sciences of the USA
Preparation of Protein Extracts and Western Immunoblot. Supernatants of penile homogenates were electrophoresed and then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia) by semidy electroblotting for 1 h (14). The membranes were blocked 1 h at room temperature with blotto-Tween (5% nonfat dry milk/0.1% Tween-20) and incubated with primary PDE5A (Calbiochem; 1:3,000), p-PDE5A (Fabgenix, Shreveport, LA; 1:2,000), eNOS (Sigma; 1:3,000), or soluble guanylyl cyclase (Sigma; 1:3,000) antibody. Bond antibody was detected with labeled anti-mouse or anti-rabbit secondary antibody (1:20,000) (Santa Cruz Biotechnology). Enhanced chemiluminescence was performed with ECL-Plus (Amersham Pharmacia), and bands were quantified by densitometry using UN-SCAN-IT software (Silk Scientific, Orem, UT).

Real-Time RT-PCR Analysis. Total RNA was isolated from mouse corpus cavernosum according to manufacturer’s instructions (Invitrogen). Real-time quantitative PCR was performed by using a 7900HT Sequence detection system (Applied Biosystems, Foster City, CA). Duplicates of each sample were subjected to reverse transcription (48°C for 30 min) and standard multiplex real-time PCR (95°C for 10 min followed by 50 cycles of 95°C for 15 sec and 60°C for 60 sec) using TaqMan One-Step RT-PCR Mastermix Reagents and primers and probes designed by using PRIMER EXPRESS (Applied Biosystems). The sense (S) and antisense (AS) PCR primer sequences are: mPDE5A (S), 5′-GCG AAG CAG ATG GTG ACA TTA-3′; mpDE5A (AS), 5′-GAT AAA GCC TGG AGC TCT CTT GGT-3′; β-actin (S), 5′-AAC CGG AGA ATG ACC CAC ATC ATG TTT-3′; β-actin (AS), 5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′. RT-PCR results were normalized for β-actin gene expression.

PDE5 Enzyme Activity Assay. Total low Km cGMP phosphodiesterase activity was assayed at 1 μM per liter of substrate by using a fluorescence polarization assay (Molecular Devices) under linear conditions with and without PDE5A inhibitor (sildenafil 0.1–1 μM, or tadafalil 50 nM) or 3-isobutyl-1-methylxanthine (50 μM). PDE-assays at 1 μM cGMP detected several high-affinity cGMP-PDEs (PDE5A, PDE9A) and dual specificity PDEs (e.g., PDE1C, PDE5A, PDE10A, and PDE11A) (15).

NOS Enzyme Activity Assay. NOS activity was assayed by radiolabelled L-arginine to L-citrulline conversion, as described (12, 16). Measurements were performed in the presence (constitutive NOS activity) or absence (inducible NOS activity) of calcium.

Tissue cGMP Levels. Quantitative assays for cGMP were performed by using a commercial enzyme immunoassay kit (Amersham Pharmacia). For penile cGMP content, frozen cavernosal tissue was homogenized in 6% trichloroacetic acid (1 ml of trichloroacetic acid per 100 mg of tissue), centrifuged, and extracted with water-saturated diethyl ether (12). In studies to determine change in cGMP level in response to maximal neurostimulation, mice excluded from previous physiologic erection studies were subjected to cavernous nerve electrical stimulation for 1 min at 6 V, and penes were snap frozen in liquid nitrogen immediately after stimulation.

In Vitro Corpus Cavernosum Tissue Culture. The penile shaft including crura without previous exposure to electrical or pharmacologic stimuli was removed after euthanasia of WT mice. The penes were cleared of adventitia, and the corpora cavernosa were then split with a scalpel. The cavernosal strips were then immersed in standard Krebs solution (pH 7.4) bubbled with 95% O2 at 37°C. After 30-min equilibration, strips were exposed to carbachol (CCCh, 10 μM) to stimulate the release of NO from the endothelium. After 10 min of exposure to CCCh, l-nitroarginine methyl ester (l-NAME) was added to the bath, and cavernosal strips were exposed at various time points (10, 30, 60, and 90 min) and immediately frozen in liquid nitrogen for later analysis for phosphorylated PDE5A (p-PDE5A), total PDE5A (t-PDE5A), soluble guanylyl cyclase, and β-actin protein expression. In a separate series of experiments, cavernosal strips were exposed to l-NAME (30 min) and subsequently treated with DEA/NO (60 and 90 min) before protein expression analysis.

Results and Discussion

Implausible although it may seem, genetically modified mice lacking eNOS (eNOS−/−) or combined nNOS and eNOS (nNOS−/−, eNOS−/−) isoforms display priapic activity and pronounced erectile responses to electrical stimulation of the cavernous nerve as well as variably phasic and prolonged erections after discontinuation of neurostimulation (ref. 17 and Fig. 1a). We evaluated penile erection comparatively in these mutant mice, mice lacking the nNOS (nNOS−/−) isoform, and WT mice by monitoring ICP in response to neurologic and pharmacologic eregogenic stimuli. All animal groups displayed penile erection in response to cavernous nerve electrical stimulation (Fig. 1b). In both nNOS−/− and WT mice, ICP increased incrementally over a standard voltage range with a maximal response at 6 V. However, eNOS−/− and double mutant mice were highly responsive to stimulation even at low voltage, achieving maximal penile erection with a 1-V stimulus. Similarly, all animal groups achieved erection in response to direct intracavernosal injection of the NO donor DEA/NO (Fig. 1c). However, although ICP increased incrementally with increasing drug dosages in all animals, it was distinctly more substantial in both eNOS−/− and double mutant mice than in nNOS−/− and WT mice. (Additional studies relating to the integrity of the nonessential cAMP-dependent mediatory pathway for erection in NOS mutant mice are described in Supporting Text and Table 1, which is published as supporting information on the PNAS web site.)

We conducted additional erection pharmacostimulation studies by using the PDE5 inhibitor sildenafil citrate. Intracavernosal injections of this drug produced dose-related increases in ICP in nNOS−/− and WT mice, whereas ICP increases were largely attenuated in both eNOS−/− and double mutant mice (Fig. 1d). These results suggested that eNOS−/− and double mutant mice, distinct from nNOS−/− and WT mice, lack a basal level of inhibitable PDE5 activity in their penes from which to induce erectile responses.

To investigate the biochemical basis for the erectile function of the mutant mice, we initially evaluated residual NOS enzyme activity in lysates of penile tissue by monitoring the conversion of arginine to citrulline (Supporting Text). Constitutive (calcium-dependent) NOS activity was reduced substantially in all NOS mutant mice groups, most profoundly so in eNOS−/− and double mutant mice, in contrast to WT mice (Table 2, which is published as supporting information on the PNAS web site). Calcium-independent conversion of arginine to citrulline was not significantly different among animal groups, confirming that the inducible NOS isoform (iNOS or NOS 2) is unaltered in the mutant mice (Table 2).

We proceeded to measure basal and neurostimulated concentration levels of cGMP in penes of the mutant mice. Under unstimulated baseline conditions, cGMP levels in nNOS−/− mice were significantly higher than levels in WT mice, whereas levels in eNOS−/− and double mutant mice were reduced to less than half of WT levels (Fig. 2a). Immediately after maximal cavernous nerve electrical stimulation, the absolute change in cGMP levels above baseline values was significantly greater by >2-fold in eNOS−/− and double mutant mice than in nNOS−/− and WT mice (Fig. 2a). These results suggested that either the production of cGMP is elevated or its catabolism is hindered expressly in eNOS−/− and double mutant mice.
To distinguish these possibilities, we performed protein immunoblotting of penile tissue for soluble guanylyl cyclase (Supporting Text) and PDE5, respectively, showing that whereas protein expression of soluble guanylyl cyclase remained constant among all groups (Fig. 4, which is published as supporting information on the PNAS web site), PDE5 protein levels were significantly reduced in eNOS−/− and double mutant mice in contrast to nNOS−/− and WT mice (Fig. 2b). The reduction in PDE5 protein expression in eNOS−/− and double mutant mice coincided with the reduction in PDE5 gene expression in these groups (Fig. 2b). We further investigated the derangement of PDE5 by evaluating the protein expression of the phosphorylated (activated) form of the enzyme and its biochemical activity, which similarly showed 75–80% reductions in eNOS−/− and WT mice (Fig. 2b). The reduction in PDE5 protein expression and PDE5 activity coincided with the reduction in PDE5 gene expression in these groups (Fig. 2b). The more marked decline in the functionally activated form of PDE5 relative to its total expression may be explained by diminished allosteric binding by cGMP required for the activated conformational state of PDE5 (18).

To further test the hypothesis that the down-regulation of PDE5A generally applies to the pathophysiology of priapism, and is not merely a peculiarity of the NOS mutant mice, we performed relevant biochemical studies in transgenic sickle cell mouse because such mouse models are reported to display a priapism phenotype (ref. 19 and Supporting Text). Penes of homozygote mutant mice (SS−/− mice) showed significantly lower NOS activity and PDE5 activity than those of heterozygotes (SS+/− mice) or WT mice (Fig. 5, which is published as supporting information on the PNAS web site).

The specificity of the NO depletion leading to changes in PDE5 biology in the penis was supported by in vitro mouse corpus cavernosum tissue studies in which we evaluated the effects of NO exposure and withdrawal on phosphorylated PDE5 expression (Supporting Text). We found that cavernosal tissue from WT mice incubated with the nonselective NOS inhibitor L-nitroarginine methyl ester yielded a time-dependent reduction in phosphorylated PDE5 expression, whereas soluble guanylyl cyclase expression was unchanged (Fig. 6, which is published as supporting information on the PNAS web site). However, after incubation of this preparation with DEA/NO, expression levels of phosphorylated PDE5 were reversibly increased (Fig. 7, which is published as supporting information on the PNAS web site).

The reduced basal but markedly elevated neurostimulated penile concentrations of cGMP, coupled with the reduced protein and gene expressions and activity levels of PDE5 in penes of eNOS−/− and double mutant mice, which display excessive erectile responses to erectogenic stimuli, imply that eNOS profoundly affects the function of downstream effectors of the NO signaling pathway controlling penile erection. We wondered whether restoration of eNOS in penes of eNOS mutant mice by gene transfer would reverse these biochemical changes and, to some extent, correct the erectile abnormalities. Using eNOS−/− mice, we applied in vivo gene transfer of eNOS to the corpora cavernosa by using an adenoviral vector driven by a CMV promoter (20). Constitutive NOS activity in penes of these transfected mice was increased without effect on inducible NOS activity, and eNOS protein expression in their penes resembled that of WT mouse penes (Supporting Text). Additionally, phosphorylated PDE5 protein expression and PDE5 activity levels normalized somewhat in these mice when compared to eNOS−/− mice transfected with reporter gene only (Fig. 3d).
Accompanying these changes was the normally moderate increase in cGMP levels in response to cavernous nerve electrical stimulation (Supporting Text).

In vivo erectile function studies revealed differences between eNOS<sup>−/−</sup> mice transfected either with eNOS or reporter gene (Fig. 3a). eNOS<sup>−/−</sup> mice transfected with eNOS exhibited a significant reduction in supranormal erection as determined by the area under the response curve (AUC) in response to intracavernosal injection of DEA/NO as well as an emergent erectile response to intracavernosal injection of sildenafil (Fig. 3b). Furthermore, the detumescence phase of neurostimulated erection, represented by the area under the response curve (AUC) after termination of cavernous nerve electrical stimulation, was shortened in eNOS<sup>−/−</sup> mice transfected with eNOS compared to that of eNOS<sup>−/−</sup> mice transfected with reporter gene, whereas detumescence in the presence of sildenafil in the eNOS-transfected mice was extended, resembling the effect observed in WT mice (Fig. 3c). These results suggested that mutant mice deficient in eNOS possess a delayed breakdown of cGMP after neurostimulated erection ceases, whereas eNOS restoration in these mice allows them to recover PDE5 activity necessary for the potentiation of neurostimulated erection by sildenafil. Further evidence for the PDE5 dysregulation in eNOS-deficient mutant mice is provided by the recovery of cGMP hydrolytic PDE5 activity after an episode of neurostimulation in penes of eNOS<sup>−/−</sup> mice transfected with eNOS compared to eNOS<sup>−/−</sup> mice transfected with reporter gene (Fig. 3d).

These results, taken together, emphasize the importance of eNOS and its effect on downstream mediators of the NO signaling pathway in the regulation of penile erection. The findings suggest that eNOS exerts a constant stimulus for the production of cGMP and consequently fulfills a requirement for PDE5 regulation. The homeostatic role demonstrated here for eNOS adds to our recent findings that eNOS plays a major role in the full achievement and maintenance of the erectile response (14). By what mechanism does eNOS affect PDE5 regulation in the penis? It would appear that depleted endothelial NO in the penis yields a state of PDE5 down-regulation. The effect apparently occurs on a gene transcriptional level and results in a restricted amount of PDE5 available for hydrolysis of cGMP. This characterization fits with the nitrate tolerance mechanism, but in a converse manner. The nitrate tolerance mechanism describes a compensatory biological response exerted by PDE5
to control nitrate overstimulation in smooth muscle, much as the concentrated NO treatment of platelets and aortic tissue in vitro is associated with an attenuated cGMP effect because of increased PDE5 activity (21). Accordingly, our data suggest a reversed nitrate tolerance mechanism whereby PDE5 is compensatorily down-regulated in response to long-standing NO understimulation.

The priapic behavior in eNOS-deficient mutant mice can be explained by a physiologically relevant supersensitization to cGMP after intense erectile stimulation. As a result of PDE5 dysregulation, the abundance of the cyclic nucleotide generated locally after an episode of neurostimulation produces unrestrained erectile tissue relaxation. A similar phenomenon conceivably occurs in humans with idiopathic stuttering and other priapism variants. In individuals with relative PDE5 deficiency, heightened psychogenic or reflexogenic neurostimulation may precipitate persistent erection from local cGMP supersensitivity. Known precipitants of priapism include rapid eye movement sleep, lengthy sessions of genital stimulation, and genital manipulation occurring during urologic surgery under anesthesia (1).
The understanding that PDE5 dysregulation may account for priapism has clinical implications. As a compartment syndrome, priapism often produces anoxic erectile tissue destruction and consequently devastates many affected men by causing complete erectile inability. Reactive surgical blood drainage procedures are often unable to preserve erectile function, urging the development of therapies that can be offered preventatively, particularly in high risk individuals or in those who are predisposed to recurrences. Our studies suggest that focus should be given to PDE5 as a molecular target for treating the disorder. One pharmacotherapy that seems potentially advantageous in an acute setting would be the local application of an agonist for binding sites on the enzyme to render it especially active (18). Another possibility for use in the long term would be the repeated administration of an NO donor to the penis that may actually boost PDE5 expression and activity by induction of nitrative tolerance. Equally counterintuitive is the consideration of eNOS gene transfer to the penis, which offers potential therapeutic utility by restoring regulatory balance associated with NO signaling of penile erection.

We thank J. M. Hare and S. H. Snyder for helpful discussions and L. Hsu (Thomas Jefferson University, Philadelphia) for providing SS mice. We acknowledge the technical assistance of B. Musicki, M. Kramer, and M. A. Palese. This work has been supported by National Institutes of Health/National Institute for Diabetes and Digestive and Kidney Diseases Grant DK 02568 (to A.L.B.) and National Institute of Health Heart, Lung, and Blood Institute Grants P50-HL52307, HL47511, and P01-HL59408 (to D.A.K.). H.C.C. is a recipient of the Shin Chun-Wang Young Investigator Award from the American Physiological Society.