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Endothelial Dysfunction in Erectile Dysfunction: Role of the Endothelium in Erectile Physiology and Disease

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Erectile dysfunction (ED) is defined as the consistent inability to obtain or maintain an erection for satisfactory sexual intercourse. Basic science research on erectile physiology has been devoted to investigating the pathogenesis of ED and has led to the conclusion that ED is predominately a disease of vascular origin. The incidence of ED dramatically increases in men with diabetes mellitus, hypercholesterolemia, and cardiovascular disease. Loss of the functional integrity of the endothelium and subsequent endothelial dysfunction plays an integral role in the occurrence of ED in this cohort of men. This communication reviews the role of the vascular endothelium in erectile physiology and the influence of endothelial dysfunction in the pathogenesis of ED. Future pharmacological and gene therapy interventions to restore endothelial function may represent exciting new therapeutic strategies for the treatment of ED.

Penile erection is a neurovascular phenomenon that depends upon neural integrity, a functional vascular system, and healthy cavernosal tissues (Giuliano et al, 1995). Normal erectile function involves 3 synergistic and simultaneous processes: 1) neurologically mediated increase in penile arterial inflow, 2) relaxation of cavernosal smooth muscle, and 3) restriction of venous outflow from the penis. The corpus cavernosum of the penis is composed of a meshwork of interconnected smooth muscle cells lined by vascular endothelium. Of note, endothelial cells and underlying smooth muscle also line the small resistance helicine arteries that supply blood to the corpus cavernosum during penile tumescence. Pathological alteration in the anatomy of the penile vasculature or impairment of any combination of neurovascular processes can result in ED. Aging is most commonly associated with ED, but a number of underlying disease processes are recognized to lead to abnormal function and responsiveness of the penile vascular bed.

ED was believed to be a psychological condition; however, in the past 2 decades, authorities have recognized that the majority of patients' erectile failure can be attributed to an organic etiology. ED may result from neurologic, arteriogenic, veno-occlusive, or cavernosal impairments and is therefore associated with vascular risk factors such as atherosclerosis, hypertension, hypercholesterolemia, diabetes mellitus, and cigarette smoking (Feldman et al, 1994; Laumann et al, 1999; McKinlay, 2000). Because ED is highly prevalent in men with cardiovascular disease, and because cardiovascular disease is well known to be associated with endothelial dysfunction, one can infer that endothelial dysfunction of the penile vascular tree may contribute to impairments in erectile function. Therefore, it has been hypothesized that endothelial dysfunction can result in ED (Maas et al, 2002; Solomon et al, 2003). Recent clinical and basic science investigations on aging, diabetes, hypercholesterolemia, and hypertension have shown that endothelial dysfunction is a major contributing factor to penile vascular pathology.

The following review examines the role of the vascular endothelium in erectile physiology and demonstrates the importance of the endothelium in normal erectile physiology and how impairments in endothelial function can cause deleterious effects on erectile function.

Endothelial Cell Function and Dysfunction

The vascular endothelium not only serves as a passive barrier for the arterial and venous blood, but also plays a pivotal role in modulating vascular tone and blood flow in response to humoral, neural, and mechanical stimuli. Furchgott and Zawadzki (1980) first reported the obligatory role of the endothelium in regulating local and basal control of vessel tone. It is now widely accepted that the vascular endothelium has a fundamental role in the regulation of vascular tone in the circulation by releasing a variety of factors that affect the contractile and relaxatory behavior of the underlying vascular smooth muscle. The actions of the endothelium are not limited to regulating vascular tone, rather, they also play a pivotal role in the regulation of inflammation, platelet aggregation, vascular

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smooth muscle proliferation, and thrombosis (Behrendt and Ganz, 2002).

The endothelium responds to chemical and hormonal signals as well as to physical hemodynamic changes caused by alterations in blood flow and shear stress by releasing mediators that modulate the tone of the underlying smooth muscle layer. In certain disease states, disruption of the functional integrity of the vascular endothelium plays an integral role in the ability of the endothelium to respond to local hemodynamic changes and paracrine and autocrine factors, a condition referred to as endothelial dysfunction (Cai and Harrison, 2000; Behrendt and Ganz, 2002; Maxwell, 2002). The regulatory role of the endothelium becomes attenuated during endothelial dysfunction, whereby there is either a decrease in responsiveness to vasodilator mediators or an increase in sensitivity to vasoconstrictors. Endothelial dysfunction refers to several pathological conditions, including altered anticoagulation and anti-inflammatory activities, impaired modulation of vascular growth, and dysregulation of vascular remodeling (Mombouli and Vanhoutte, 1999). However, the term endothelial dysfunction is most commonly used to refer to decreases in endothelium-dependent smooth muscle relaxation caused by a loss of or increased destruction of nitric oxide (NO) bioactivity in the vasculature.

The decrease in NO bioavailability in endothelial dysfunction may be caused by reductions in the enzyme endothelial NO synthase (eNOS, NOS3); a lack of substrate or cofactors for eNOS; alterations in intracellular signaling such that eNOS is not appropriately activated or uncoupled; or accelerated degradation of NO by reactive oxygen species (ROS), such as superoxide anion. Importantly, in endothelial dysfunction, responses to the endothelium-independent vasodilator sodium nitroprusside (SNP) are usually unaltered, indicating that dysfunction arises from abnormal NO production or release from the endothelial cells. However, it is conceivable that other factors, such as superoxide anion, may be preventing NO from eliciting the normal vasodilator response. This condition causes a disruption in the balance of the vasoactive mediators, whereby the role of vasoconstrictors becomes even more prominent and the role of vasodilators diminishes, thereby affecting normal vascular tone.

NO-Dependent Penile Erection: Role of the Endothelium

Various cellular processes are regulated through the release of NO from the endothelium, platelets, vascular smooth muscle cells, neurons, and other cell types (Ignarro et al, 1999). NO has many important physiological roles, including neurotransmission, regulation of vascular tone, immunomodulation, cell-mediated cytotoxicity against pathogens and tumor cells, and penile erection (Burnett, 1995; Ignarro et al, 1999; Bogdan, 2001). In addition to NO's direct toxic effects, it has been shown to exhibit an inhibitory effect on smooth muscle cell proliferation and collagen synthesis.

The formation of NO and L-citrulline from its substrate L-arginine occurs in most tissues of the body. The enzyme that catalyzes this reaction in cells and neurons is termed NO synthase (NOS). This enzyme uses reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinculeotide, flavin mononucleotide, and tetrahydrobiopterin (BH_4) as cofactors and heme as a prosthetic group. The constitutive forms of the enzyme, neuronal NOS (nNOS; NOS1) and eNOS, are coupled to Ca^{2+} and calmodulin and are the principal NOS isoforms involved in the induction of penile erection (Ignarro et al, 1990; Burnett et al, 1992; Rajfer et al, 1992), whereas inducible NOS (iNOS; NOS2) is independent of Ca^{2+} and calmodulin and requires new protein synthesis (Alderton et al, 2001). eNOS is predominately membrane bound, whereas nNOS is limited to the cytosol of central and peripheral neurons, although its mRNA is also localized to the skeletal muscle (Pollock et al, 1991).

The concentrations of NO are continuously fluctuating at very low levels throughout the vascular system and are controlled predominately by eNOS. The constitutive enzymes eNOS and nNOS are regulated predominately at the posttranslational level, whereas iNOS is expressed in response to an appropriate stimulus (eg, cytokines, inflammation) or transcriptional factors (nuclear factor kappa B; NF-κB) (Forstermann and Kleinert, 1995). Recently, the subcellular localization of eNOS to distinct microdomains of the plasma membrane, its interaction with the protein caveolin-1, and the phosphorylation state of specific serine and threonine residues of the enzyme have been found to play an integral part in the posttranslational regulation of eNOS activity (Feron et al, 1996; Fleming and Busse, 1999; Michell et al, 1999; Boo et al, 2002; Goligorsky et al, 2002). NO produced by 1) eNOS from endothelial cells lining the cavernosal smooth muscle cells and resistance helicine arteries in response to shear stress, 2) agonist-induced activation by acetylcholine released from cholinergic nerves, and 3) nNOS activity in nonadrenergic, noncholinergic (NANC) neurons is involved in signaling events that regulate neurotransmission and penile vascular tone.

The most important physiological target of NO in the penis is the heme moiety of soluble guanylate cyclase (Mizusawa et al, 2002). NO diffuses to adjacent smooth muscle cells stimulating guanylate cyclase. This interaction converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which induces a substantial increase in intracellular cGMP, causing smooth muscle relaxation. Its action is primarily through the cGMP-dependent kinase I (cGKI, PKG), which alters intracellular Ca^{2+} levels by reducing Ca^{2+} channel activity

and opening Ca^{2+}-dependent K^+ channels, leading to hyperpolarization of the smooth muscle cell (Christ et al, 1999). PKG can also phosphorylate other proteins to affect Ca^{2+} channels or lead to an alteration of the phosphorylation state of the myosin light chain (MLC) (Mills et al, 2002b). These second messengers reduce intracellular Ca^{2+} via Ca^{2+} sequestration and extrusion and activation of MLC phosphatases. The NO/cGMP-dependent smooth muscle relaxation results in entry of blood and engorgement of the corpus cavernosum and penile erection. The physiological actions of cGMP are terminated by the hydrolysis of the 3′5′ bond by the type 5 phosphodiesterase. The importance of the protein kinase cGKI in the erectile process was established in cGKI-deficient mice (Hedlund et al, 2000a). These knockout mice are unable to reproduce and have impaired cavernosal smooth muscle relaxation in response to neuronal and endothelial derived NO and exogenous NO.

Until recently, the defined role of eNOS and nNOS in the regulation of NO-dependent penile erection has been a subject of great debate. Mice lacking the genes for both eNOS and nNOS were still able to exhibit normal erectile function and mating behavior (Burnett et al, 1996, 2002). The first explanation proposed for the maintenance of the NO-dependent erectile response in nNOS-alpha -/- mice was a compensatory up-regulation of eNOS to fulfill insufficient nNOS expression. A more recent explanation for the intact NO-dependent erectile response in these mice is the existence of nNOS gene variants resulting from alternative mRNA splicing of the nNOS-beta and nNOS-gamma alternative translation in exon 1 (Burnett, 2000; Gonzalez-Cadavid et al, 2000). eNOS -/- mice demonstrate normal erectile function to electrical stimulation of the cavernosal nerve even when their penises have only 60% of the NOS activity compared with wild-type mice (Burnett et al, 2002). Of particular interest, studies from eNOS -/- mice have shown direct physiologic evidence for the contribution of eNOS in mediating cholinergic-stimulated penile erections, thus documenting the importance of cholinergic stimulation and agonist-induced activation of the endothelium in cavernosal smooth muscle relaxation and erection (Burnett et al, 2002). Thus, transgenic mice studies with targeted deletion of the eNOS and nNOS genes have further elucidated the role of these NOS isoforms on the regulation of penile erection and have reinforced our current understanding of the importance of NO regulatory control of penile erection.

NO production from nNOS in the NANC nerves innervating the penis is essential for the initiation of cavernosal smooth muscle relaxation and subsequent erection. The relative importance of endothelial-derived NO from eNOS in the endothelial cells of the corpus cavernosum and arteries supplying the penis has recently been elucidated. Immunohistochemical and molecular probes for eNOS have demonstrated its presence in the trabecular lining of the corpus cavernosum and in the small intracavernosal resistance helicine arteries of the penis (Bloch et al, 1998; Hedlund et al, 1999; Bivalacqua et al, 2001a; Gonzalez et al, 2001; Mizusawa et al, 2001; Stanarius et al, 2001). Both human and animal studies have demonstrated that the corpus cavernosum is capable of relaxing in the presence of endothelium-dependent agonists (acetylcholine, carbachol, bradykinin) and impairment of endothelium-dependent cavernosal smooth muscle relaxation in vitro occurs in vascular-associated diseases, such as diabetes mellitus, hypertension, and hypercholesterolemia (Saenz de Tejada et al, 1989; Gur et al, 2000; Behr-Roussel et al, 2002, 2003). However, what role does endothelial-derived NO play in the regulation of normal penile erection in vivo?

In the past 5 years there has been significant evidence supporting the vital role of endothelial-derived NO from eNOS in the regulation of penile erection both in normal physiology and in pathological disease states. Our current understanding on the mechanism for initiation and maintenance of penile erection is that penile erection is elicited by neural signals form the spinal cord, which stimulates nNOS activity and increases the production of NO from NANC nerves, thereby causing an increase in blood flow to the cavernosal tissue (Moreland et al, 2001b). eNOS is then activated by a shear stress/mechanical mechanism by increased blood flow from the arteries supplying the corpora and expansion of the sinusoidal spaces of the corpora. The continued shear stress on the endothelial lining of the intracavernosal smooth muscle cells and arteries continues to produce endothelial-derived NO, which maintains the tumescence phase of penile erection (Figure 1).

Hurt and colleagues (2002), using selective pharmacological inhibitors and eNOS knockout mice, first showed that penile erection-dependent processes to cavernosal nerve stimulation and drug-induced relaxation of the corpus cavernosum are mediated by phosphatidylinositol 3-kinase (PI3-kinase) and activation of the serine/threonine protein kinase Akt. This pathway phosphorylates eNOS to increase endothelial-derived NO (Michell et al, 1999). The use of pharmacological inhibitors of PI3-kinase in the penis of rats and eNOS -/- mice demonstrated that these inhibitors were able to reduce erections to electrical nerve stimulation and intracavernous papaverine. This signaling pathway was furthermore shown to be responsible for sustained NO production via a PI3 kinase/Akt-dependent activation of eNOS with subsequent increases in endothelial-derived NO and maintenance of maximal erection (Figure 1).

The application of targeted genes involved in the erectile process has further enabled researchers to study the mechanisms involved in the pathophysiology of ED-as-

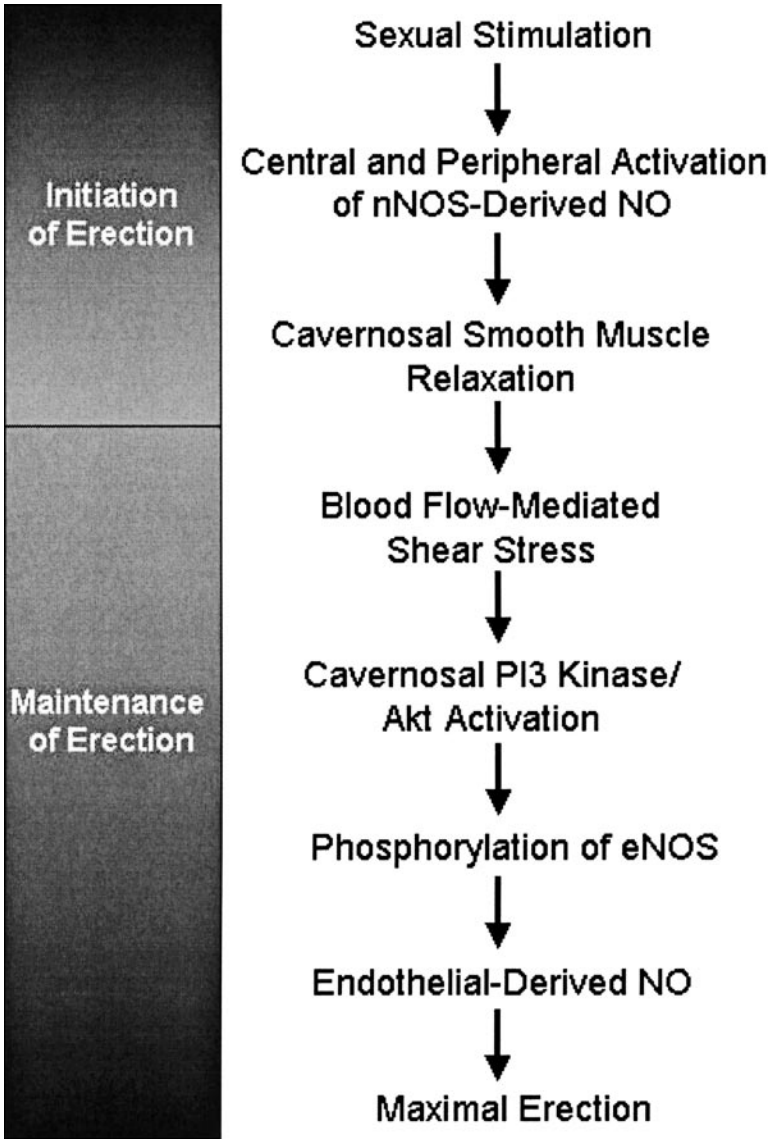

Figure 1. Schematic diagram of the independent roles of nNOS and eNOS in the initiation and maintenance of the erectile response. (Adapted from Hurt et al, 2002.)

sociated conditions, such as aging, diabetes, and hypercholesterolemia. Both the natural aging process and diabetes cause significant impairment in erectile function that is contributed to numerous factors both in the central and in the peripheral nervous system as well as at the end organ. Most notably, changes in neural integrity of the cavernosal nerve and pelvic plexus as well as in endothelial cell function are well recognized. ED associated with these conditions is multi-factorial, but most authorities recognize that there is an overall reduction in NO biosynthesis (Sullivan et al, 1999; Maas et al, 2002). Until recently, the independent role of eNOS in these disease processes was unknown. By using adenoviral gene transfer of eNOS to the corpus cavernosum, it was determined that this NOS isoform was capable of restoring diminished erectile function in aged rats and rats with diabetes independent of nNOS expression (Champion et al, 1999; Bivalacqua et al, 2000b; Bivalacqua and Hellstrom, 2001; Bivalacqua et al, 2003b, in press). These studies have demonstrated that in disease states, in which eNOS expression is reduced or unaltered, overexpression of this NOS isoform is capable of restoring erectile function to cavernosal nerve stimulation via increased endothelial-derived NO biosynthesis and cavernosal cGMP levels. Importantly, eNOS gene therapy has no effect on nNOS protein or gene expression, in that restoration of erectile function is solely dependent upon eNOS expression and endothelial-derived NO bioactivity. These studies docu-

ment the importance of eNOS in the maintenance of the erectile response and its significance in pathological conditions associated with ED and endothelial dysfunction.

Endothelium-Derived Vasodilators and Vasoconstrictors in the Penis

A number of review articles address the mechanisms and pharmacology of penile erection (Bivalacqua et al, 2000a; Christ, 2000; Andersson, 2001b, 2003). The following section contains an overview of the endothelium-derived vasodilator and vasoconstrictor agents involved in the physiology of penile erection.

Cavernosal smooth muscle cells in the penis are predominately found in the contracted state with minimal blood flowing through the cavernous sinuses. The balance between known contractile systems (RhoA/Rho-kinase, α-adrenergic, endothelin, angiotensin, thromboxane A_2) and vasodilatory second-messenger systems (adenylate cyclase-cyclic AMP and guanylate cyclase-cyclic GMP) determines the overall tone of corpora cavernosa smooth muscle of the penis (Andersson, 2001a; Mills, 2002). This balance is controlled by both central and peripheral mechanisms and involves a plethora of neurotransmitters acting through various signal transduction pathways.

Endothelial cells actively regulate basal vascular tone and vascular reactivity in physiological and pathological conditions by responding to mechanical forces and neurohumoral mediators with the release of a variety of relaxing and contracting factors. In the corpus cavernosum, the vascular endothelium and cavernosal arteries are a source of vasorelaxing factors such as NO; prostacyclin (PGI_2); the not-yet identified endothelium-derived hyperpolarizing factor (EDHF); and the vasoconstrictor factors angiotensin II (Ang II), endothelin-1 (ET-1), and Rho-kinase. These endothelium-derived factors have a regulatory influence on cavernosal vascular tone. Normally, these factors act in concert to elicit an overall beneficial effect on cavernosal smooth muscle function, which is crucial in response to changes in blood flow, shear stress, and agonists in order to maintain physiological homeostasis throughout the penile vascular bed. The critical balance of vasodilators and vasoconstrictors is normally maintained during health and quickly responds to changes in blood flow and other factors. Decreases in NO-, prostaglandin-, and EDHF-mediated responses have been shown to be involved in cardiovascular diseases related to endothelial dysfunction, and increases in responses to Ang II and ET-1 have also been implicated (Harrison, 1997; De Vriese et al, 2000). Evidence now exists to demonstrate that this also occurs in the penile vascular bed.

NO—The principle mediator of cavernosal smooth muscle relaxation is NO released by the NANC neurons innervating the penis and by cavernosal endothelial cells (Burnett, 1995). NO is released from endothelial cells under the influence of agonists, such as acetylcholine from cholinergic neurons in the penis, and mechanical forces, such as shear stress caused by pulsital blood flow in the sinusoidal spaces of the corpus cavernosum (Hedlund et al, 2000b). NO diffuses to the underlying smooth muscle cells where it activates the soluble form of guanylate cyclase elevating intracellular levels of cGMP and the activity of cGKI protein kinase. The NO/cGMP-signaling cascade reduces contractile activity and promotes cavernosal smooth muscle relaxation (see previous section).

Prostaglandin—NO can activate prostaglandin (PGE) synthesis in vivo. During shear stress blood flow in the penis, another mechanism involved to enhance cavernosal smooth muscle relaxation is activation of PGE synthesis via a NO-dependent mechanism (Ballermann et al, 1998). The vasodilators PGE and PGI_2 are primarily produced by endothelial cells in the vasculature. PGI_2 is the most abundant arachidonic acid product generated in vascular tissues (McNamara et al, 1998). Both isoforms of cyclooxygenase, COX-1 and COX-2, can convert arachidonic acid to PGH_2. PGH_2 is subsequently converted to PGI_2 by the action of PGI_2 synthase (PGIS) and PGE_2 by PGE_2 synthase. PGE synthesized by endothelial cells in the corpus cavernosum in response to mechanical shear stress blood flow in the penis binds to specific PGE (EP) receptors on smooth muscle cells (Traish et al, 1997; Meghdadi et al, 1999). Activation of EP receptors by PGE increases intracellular levels of cAMP via activation of adenylate cyclase, causing a reduction in intracellular levels of Ca^{2+} and cavernosal smooth muscle relaxation (Moreland et al, 2001a). Both PGE_2 and its derivative PGE_1 are potent vasorelaxing agents in human corpus cavernosum smooth muscle. There are 4 distinct EP receptors ($EP_{1–4}$), and all 4 are expressed in the corpus cavernosum (Angulo et al, 2002). EP_2 and EP_4 are G-protein–coupled receptors and are responsible for an increase in cAMP synthesis in response to exogenous PGE_1 administration to the human penis and in cultured cavernosal smooth muscle cells. PGE_1 is a potent vasodilator of the penile vascular bed and is a highly efficacious local agent for the treatment of ED (Leungwattanakij et al, 2001).

EDHF—The currently unidentified EDHF likely plays an important role in erectile physiology. EDHF is released by endothelium-dependent agonists or shear stress and hyperpolarizes the underlying smooth-muscle–inducing relaxation by decreasing intracellular levels of Ca^{2+} within the smooth muscle cells (Busse et al, 2002). This mechanism involves decreasing the opening probability of the voltage-dependent Ca^{2+} channels and reducing the turnover of intracellular phosphotidylinositides. EDHF-mediated responses increase as the vessel size decreases (Mombouli and Vanhoutte, 1997). It was once believed that the final common pathway leading to smooth muscle hyperpolarization by EDHF required the opening of po-

tassium channels on the smooth muscle cell membrane; however, this hypothesis has been modified in light of more recent experimental observations.

Current evidence suggests that EDHF is formed after an increase in endothelial Ca^{2+}, either induced by an agonist (acetylcholine, bradykinin) or shear stress that triggers the synthesis of a cytochrome P450 metabolite, which is essential for the subsequent EDHF-mediated response (Fisslthaler et al, 1999; Fleming, 2001). Epoxyeicostatrienoic acid (EETs) is an arachidonic-acid–derived product of cytochrome P450 epoxygenases that appears to play an important role in the regulation of vascular homeostasis (Fleming, 2001; Zhang et al, 2001). EETs are reported to be potent vasodilators in a number of peripheral vascular beds. In human coronary arteries, inhibitors of cytochrome P450 2C block EDHF-mediated responses, and EETs relax coronary arteries by hyperpolarizing smooth muscle cells through a K-channel–dependent mechanism (Miura and Gutterman, 1998; Miura et al, 1999). However, the role of EDHF-mediated cavernosal smooth muscle relaxation has not been determined until recently. Angulo et al (2003) revealed that an EDHF-mediated relaxation of human penile resistance arteries exists, which is resistant to NOS and COX inhibition, suggesting that EDHF may play an important role in the endothelium-dependent relaxation of the penile vascular bed. Additionally, our laboratory has found that the pharmacological inhibitor of cytochrome P450 2C, sulfaphenazole, attenuates cavernosal nerve mediated erectile responses in the rat. This suggests that a cytochrome P450 metabolite may mediate an EDHF-dependent smooth muscle effect in the penis that may contribute to the erectile response (Figure 2). Because this area of penile vascular biology is not fully elucidated, further research must be undertaken to evaluate the potential importance of this endothelium-derived relaxing factor in the regulatory control of penile erection.

Ang II—Ang II is a potent vasoconstrictor. In addition to the classical renin-angiotensin system (RAS) operating systemically, there is a functional RAS that generates Ang II locally in the vascular tissue of the penis (Kifor et al, 1997). Angiotensin receptors characterized in the penile vasculature suggest that endothelial cells in the corpus cavernosum may form a local Ang II–producing paracrine system that may modulate vascular tone by keeping the cavernosal smooth muscle cells in a constricted state (Park et al, 1997). In organ bath studies of isolated strips of corpus cavernosum, Ang II caused a dose-dependent contraction of the cavernosal tissue in vitro (Becker et al, 2001c). Elevated levels of Ang II have been noted in men with organic ED, suggesting this peptide may play a role in the pathogenesis of ED (Becker et al, 2001a).

ET-1—The endothelins (ET-1, ET-2, and ET-3) are a family of related peptides. ET-1 is a 21 amino acid peptide generated in the vascular endothelium that is recognized as a potent and sustained vasoconstrictor in the penile vasculature (Christ et al, 1995; Mills et al, 2001b). The effects of ET-1 are mediated through ET_A receptors, which are located on the underlying smooth muscle, and through ET_B receptors, which are located on the smooth muscle and vascular endothelium. ET_A receptors mediate contraction and promote growth of smooth muscle. ET_B receptors on smooth muscle also mediate contractions, whereas stimulation of ET_B receptors on the endothelium promote NO and prostacyclin-mediated vasorelaxation. Of note, ET-1 acts as a vasodilator at low doses (ET_B receptor activation) and as a vasoconstrictor (ET_A receptor activation) at high doses when administered to cavernosal strips in vitro. Basal production of ET-1 by endothelial cells of the corpus cavernosum is hypothesized to contribute to sustained cavernosal smooth muscle contraction and maintenance of penile flaccidity. Both animal and human studies have not validated this hypothesis; therefore, despite the existence of ET receptors in the penis, most whole animal experimental and human clinical data do not support a central role for ET-1 in regulating the normal erectile response (Becker et al, 2000, 2001b; Dai et al, 2000; Kim et al, 2002). Diabetic corpus cavernosum obtained from animal models and humans have shown that ET_A receptors are upregulated and ET_B receptors are downregulated, suggesting that ET-1 may be involved in the pathogenesis of diabetic ED (Sullivan et al, 1997, 1998; Chang et al, 2003). However, future studies are warranted to establish the functional significance of these cavernosal molecular changes in the pathophysiology of diabetic-associated endothelial dysfunction.

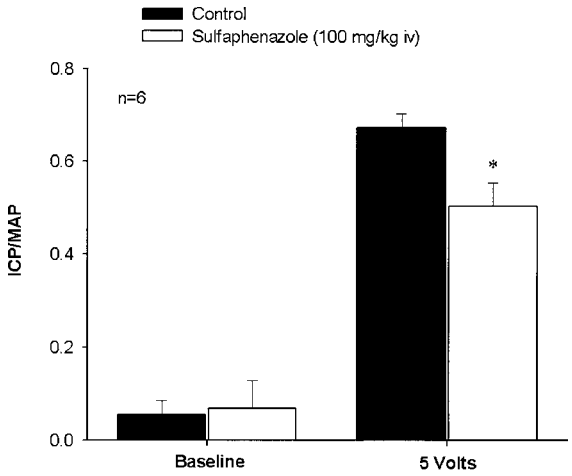

Figure 2. Bar graph depicting the voltage-dependent erectile response (ICP/MAP) after cavernosal nerve stimulation at the 5 V setting for 1 minute in control rats before and after administration of the cytochrome P450 2C inhibitor, sulfaphenazole, in a dose of 100 mg/kg IV. The in vivo erectile response was measured at baseline and 30 minutes after administration of sulfaphenazole. *n* indicates number of experiments; *, ($P < .05$) response significantly different from baseline response.

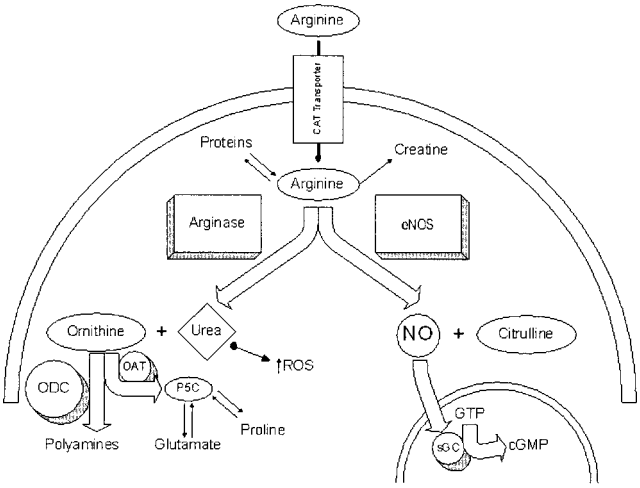

Figure 3. Arginine metabolism. L-arginine enters the endothelial cell through cationic amino acid transporter (CAT) and can react with the enzymes arginase or eNOS. When L-arginine reacts with eNOS, NO and L-citrulline is formed. NO can then bind to the soluble form of guanylate cyclase (sGC) to form cGMP. When L-arginine reacts with arginase, L-ornithine and urea are formed. Ornithine can react with ornithine decarboxylase (ODC) to form polyamines that contribute to cell proliferation or react with ornithine aminotransferase (OAT) to form pyrroline-5-carboxylate (P5C). Urea can form reactive oxygen specices (ROS) in some cell types.

Arginase—Arginine is a precursor for the synthesis of NO, urea, polyamines, creatine phosphate, and various proteins (Figure 3). This amino acid is transported from the circulation into mammalian cells by cationic amino acid transporter (CAT) isoforms (Durante, 2001). Cationic amino acids such as L-arginine are transported into cells via the y+ transport system. This systems activity is mediated by the CAT family, which is composed of 4 isoforms: CAT-1, CAT-2A, CAT-2B, and CAT-3. The existence of these CAT transporters has not been documented in the penis, but their existence is inevitable. The major site of arginine metabolism is the liver, where L-arginine generated in the urea cycle is converted to urea and ornithine by the enzyme arginase. Many additional tissues and cell types also contain the enzyme arginase, in particular endothelial cells (Li et al, 2002).

In endothelial cells, arginine is used as a substrate by both eNOS and arginase. Because both NOS and arginase use arginine as a common substrate, arginase may downregulate NO biosynthesis by competing with NOS for L-arginine (Figure 3). Thus, NO production is likely to be linked to the regulation of arginase activity (Bivalacqua et al, 2001b; Kim et al, 2001). Arginase exists in 2 isoforms: the hepatic type (arginase I) and the extrahepatic type (arginase II) (Mori and Gotoh, 2000). Recently, our laboratory has shown that endothelial cells obtained from the mouse corpus cavernosum contain both arginase isoforms (Bivalacqua et al, 2002). Additionally, by using semiquantitative RT-PCR and Western blot analysis, both arginase isoforms have been localized in the human corpus cavernosum (Bivalacqua et al, 2001b). Moreover, inhibition of arginase with 2(S)-amino-6-boronohexanoic acid (ABH) is associated with enhanced NANC- and endothelium-dependent vasorelaxation of human corpus cavernosum smooth muscle, suggesting that inhibition of arginase will increase NO biosynthesis through a NOS-dependent manner (Cox et al, 1999). Theoretically, it is feasible to regulate NO biosynthesis in both endothelial and smooth muscle cells of the penis by controlling the availability of arginine to react with NOS, and regulation of the arginase enzyme can accomplish this. Arginase activity has been shown to be upregulated in diabetic human corpus cavernosum, suggesting that the diminished erectile response caused by decreased NO production found in men with diabetes may be due to a combination of increased expression of arginase and decreased amounts of NOS nerve fibers (see section on Endothelial Dysfunction and Diabetes). Additionally, the expression of CAT transporters is also a potential determinant of the rate of L-arginine delivery to eNOS and thus can be another rate-limiting step in NO biosynthesis. Factors that affect CAT activity and the rate of transport of L-arginine in endothelial cells include the concentration of other cationic amino acids, oxidized lipoproteins, glucose, and insulin (Zharikov and Block, 1998). Thus, diabetes and hyperlipidemia may influence arginine transport in the penis and contribute to endothelial dysfunction observed in these pathological states.

RhoA/Rho-kinase—Although the mechanisms involved in the regulation of the vasorelaxation of the penile vascular bed by endothelial-derived vasodilators has been extensively studied, the endothelial-derived agents involved in the maintenance of the contracted state are only recently gaining widespread attention. Contraction of cavernosal smooth muscle is primarily mediated by the Ca^{2+}-dependent activation of MLC kinase, resulting in the phosphorylation of MLC, and subsequent actin/myosin cross-bridge formation (Figure 4). In addition, recent evidence has established the important role of Ca^{2+}-sensitization through the Ca^{2+}-independent promotion of MLC kinase or the attenuation of MLC phosphatase activity (Chitaley et al, 2001b, 2003). A principle regulator of MLC phosphatase is the serine/threonine kinase, Rho-kinase. Data from other vascular beds suggest that RhoA, a member of the Ras low molecular weight of GTP-binding proteins, mediates agonist-induced activation of Rho-kinase (Wettschureck and Offermanns, 2002; Sward et al, 2003). The exchange of GDP for GTP on RhoA and translocation of RhoA from the cytosol to the membrane are markers of its activation and enable the downstream stimulation of various effectors such as Rho-kinase. Numerous studies have established an important role for RhoA and Rho-kinase in numerous cellular responses, in-

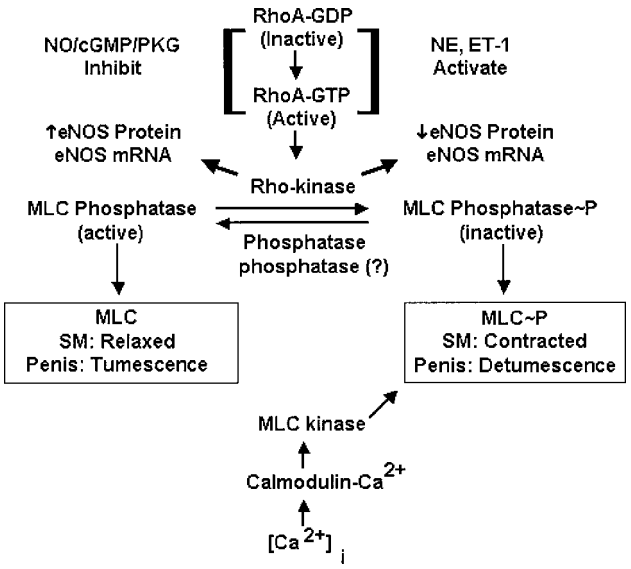

Figure 4. RhoA/Rho-kinase signal transduction pathway and its interaction with eNOS in the penile vascular bed. RhoA is activated by either agonist-induction (norepinephrine, endothelin-1) or vascular disease states (diabetes, hypertension) when it binds GTP, undergoes geranylgeranylation, and migrates to the cell membrane. Activated RhoA stimulates Rho-kinase expression, which, in turn, reduces eNOS protein and mRNA stability and catalyzes the phosphorylation (and inactivation) of myosin light chain (MLC) phosphatase. This in turns promotes cavernosal smooth muscle contraction via 2 separate mechanisms of action. When MLC phosphatase is in the active form (nonphosphorylated), it catalyzes the dephosphorylation of MLC and thereby promotes cavernosal smooth muscle relaxation. This process occurs when the downstream mediator of the NO/cGMP system, PKG, inhibits RhoA translocation to the membrane and reduces Rho-kinase expression. This results in an increase in eNOS expression and endothelial-derived NO and cavernosal smooth muscle relaxation through 2 separate mechanisms, dephosphorylation of MLC and increased eNOS expression.

cluding the contraction of smooth muscle cells (Uehata et al, 1997; Chitaley et al, 2001a). Both norepinephrine (NE) and ET-1 stimulate the GTPase RhoA in vascular smooth muscle cells, suggesting NE and ET-1 may cause vasoconstriction through a RhoA/Rho-kinase-dependent manner. Although the role of RhoA/Rho-kinase has been well outlined in numerous forms of smooth muscle, recent evidence has demonstrated its importance in the regulation of cavernosal smooth muscle tone (Chitaley et al, 2001b, 2002; Mills et al, 2001a; Rees et al, 2001). Both human endothelial cells and human corpus cavernosum smooth muscle cells grown in culture express RhoA and Rho-kinase (Essler et al, 1998; Rees et al, 2002; Wang et al, 2002; Wojciak-Stothard and Ridley, 2003).

Evidence supporting the role of Rho-kinase in the maintenance of cavernosal smooth muscle vasoconstriction has been demonstrated by administration of the Rho-kinase inhibitor, Y-27632, directly into the cavernosal sinuses of rats, which caused dose-dependent increase in intracavernosal pressure (Chitaley et al, 2001b). Additionally, adeno-associated viral gene transfer of the dominant negative RhoA mutant to the rat corpus cavernosum enhanced erectile function, suggesting that inhibition of RhoA/Rho-kinase expression in the penis can augment cavernosal smooth muscle relaxation and subsequent erectile function (Chitaley et al, 2002). Treatment with Y-27632 also potentiated voltage-dependent (NO-mediated) increases in erectile function, and inhibitors of NOS or guanylate cyclase did not block this effect (Mills et al, 2002a). Studies by Sauzeau and colleagues (2000, 2003) have demonstrated that NO and PKG can inhibit the translocation of RhoA to the membrane in the rat aorta. Additionally, SNP was found to reverse the phenylephrine (PE)-induced translocation of RhoA in the rat aorta, which is further indicative of NO's inhibitory action on RhoA activity (Chitaley and Webb, 2002). In the rat penis, NO mediates the erectile response, in part, via inhibition of the RhoA/Rho-kinase Ca^{2+}-sensitizing pathway. These data demonstrate that NO inhibits Rho-kinase activity, supporting the hypothesis that endogenous NO-mediated vasodilation may occur through the inhibition of Rho-kinase vasoconstrictor activity.

Recent evidence suggests that the RhoA/Rho-kinase signal transduction pathway is an important signal mediator of penile vascular endothelial cell function (Bivalacqua et al, 2003d). The RhoA/Rho-kinase pathway plays an important role in suppression of eNOS gene expression and enzyme activity in human endothelial cells, which results in decreased endothelial-derived NO biosynthesis (Figure 4). This mechanism may play an important role in the regulation of penile endothelial cell function and dysfunction as related to vascular diseases of the penile vasculature (see section on Endothelial Dysfunction and Diabetes).

ROS—ROS are a family of molecules produced by all aerobic cells. Oxidation of biological molecules (DNA, protein, lipids) occurs as a result of increased steady-state levels of ROS or decreased antioxidant defense mechanisms. This phenomenon is commonly referred to as oxidative stress. Many ROS possess unpaired electrons and therefore are free radicals. These include molecules such as superoxide anion, the hydroxyl radical, lipid radicals, and NO. Hydrogen peroxide and peroxynitrite are not free radicals by definition but have deleterious oxidative effects that contribute to oxidative stress in many cell types such as the vascular endothelium. Endothelial cells secrete ROS in response to shear stress, endothelium-dependent agonists (acetylcholine, bradykinin), and in various vascular disease states.

One of the most widely studied endothelial-derived ROS in the vasculature is superoxide anion. Superoxide anion is produced in a variety of cells, including neutrophils, monocytes, B-lymphocytes, platelets, mast cells, vascular smooth muscle cells, and endothelial cells (Munzel et al, 1997; Wolin et al, 2002). Several cells, most notably endothelial cells, use the membrane-associated

enzyme, NADH phosphate oxidase (NADH/NADPH), to generate superoxide anion (Munzel et al, 1999). Other potential sources of ROS in endothelial cells include lipoxygenase and COX (arachidonic acid pathway enzymes), peroxidases, cytochrome P450s, xanthine oxidase, and eNOS.

The reaction of superoxide anion and NO in the vascular endothelium or smooth muscle cells results in the formation of the highly toxic molecule, peroxynitrite (Wolin, 2000). Peroxynitrite is known to cause tissue injury, alterations in vascular tone, oxidation of cellular proteins and lipids, apoptosis, and organ dysfunction via its direct toxic effects (Beckman et al, 1990; Beckman and Koppenol, 1996). This reaction is 30 times faster than that of NO and oxyhemoglobin and 3 times faster than the dismutation of superoxide-by-superoxide dismutase (SOD). Given this rapid reaction rate, there is usually some superoxide anion reacting with NO within cells and the extracellular space. The antioxidants SOD, catalase, glutathione peroxidase, and reductase play an important role in the cellular protection against ROS (Kunsch and Medford, 1999). SOD represents a major cellular defense against superoxide anion and peroxynitrite formation by accelerating the dismutation of superoxide into H_2O_2 and molecular oxygen (Fukai et al, 2002). Three SOD isoforms have been identified, including a cytosolic copper/zinc-containing form (CuZnSOD), a mitochondrial manganese form (MnSOD), and an extracellular isoform (EC-SOD), which is also a copper/zinc-containing enzyme. Given its location, EC-SOD is hypothesized to play a critical role in modulating the redox state of the vascular interstitium and thereby preventing the pathophysiological effects of superoxide anion in the vasculature, specifically the endothelial and smooth muscle cells (Fukai et al, 2002). Both the Cu/Zn and EC isoforms have been found in the penis, predominately in the endothelial and cavernosal smooth muscle cells (Bivalacqua et al, 2003a). Conceptually, an increased level of superoxide anion in the endothelium and cavernosal smooth muscle may contribute to ED by decreasing penile NO biosynthesis and causing endothelial dysfunction as commonly observed in the vascular effects of diabetes, hypercholesterolemia, and aging. This is an important concept because cavernosal smooth muscle relaxation and ultimately penile erection depend upon the sustained production of NO by a healthy endothelium.

Endothelial Dysfunction and Oxidative Stress in the Penis

A considerable body of evidence implicates oxidative stress, in particular the reaction of NO and superoxide anion, as an important pathogenic element in the development of endothelial dysfunction in vascular diseases such as diabetes, hypertension, arteriosclerosis, and hypercholesterolemia. These vascular disorders are highly prevalent in patients with ED and have been identified as independent risk factors for ED in large, population-based studies. Increased inactivation of NO by superoxide anion in conditions of increased oxidative stress creates an imbalance that leads to a deficit of endothelial-derived NO acutely and ultimate development of endothelial dysfunction. Superoxide production can occur in the vasculature through the actions of 3 major enzyme systems: NADH/NADPH oxidases, xanthine oxidase, and eNOS. Besides reducing NO-biosynthesis, superoxide anion directly promotes a number of events, which may lead to impairments in penile erection (Jones et al, 2002). Superoxide anion can cause Ca^{2+} mobilization, thus reducing intracellular levels of Ca^{2+} in the cavernosal endothelial cells, whereas an increased production of peroxynitrite leads to generation of vasoconstricting agents and potential apoptosis of endothelial cells. These events may contribute to impairments in the penile vasculature observed in ED associated with conditions known to increase oxidative stress and superoxide anion production.

The majority of experimental data on the role of superoxide anion on corpus cavernosal and endothelial function in the penis has been studied in animal models. The effect of a superoxide anion-generating agent on in vitro NO-mediated cavernosal smooth muscle relaxation demonstrated that acetylcholine-mediated relaxation was impaired in the rat corpus cavernosum in the presence of increased production of superoxide anion, suggesting that superoxide anion can impair endothelial-derived NO in normal erectile tissue (Cartledge et al, 2000). However, the interaction of superoxide anion and NO-mediated erectile responses both in vitro and in vivo has been more extensively studied in diseased animal models of ED.

It is well known that hypercholesterolemia results in significant impairments in endothelium-dependent corpus cavernosal smooth muscle relaxation. Rabbits fed high-cholesterol diets developed impairments in corpus cavernosal endothelial smooth muscle relaxation at a time when cavernosal superoxide anion levels were significantly elevated in the hypercholesterolemic group (Kim et al, 1997). Additionally, there was a significant increase in cavernosal total CuZn levels and MnSOD levels, in order to scavenge the excess superoxide anion present as a result of hypercholesterolemia. In spite of the elevated SOD levels, there were still significant impairments in endothelium-dependent smooth muscle relaxation.

Low-density lipoprotein (LDL) peroxidation contributes to the development of atherosclerosis, and injuries to endothelial cells have a principal role in the progression of atherosclerotic lesions (Rubbo et al, 2002). Oxidized LDL (ox-LDL) is an oxidative stress-derived pathogen formed by superoxide anion and peroxynitrite and highly associated with hypercholesterolemia and atherosclerosis.

Ox-LDL has been shown to impair endothelium-dependent relaxation in the penis and may also contribute to endothelial dysfunction observed in hypercholesterolemia through an increased production of superoxide anion via uncoupling of eNOS or a reduction in the eNOS cofactor BH_4 (Ahn et al, 1999). Additional studies are warranted to delineate the exact mechanisms involved in a superoxide anion-dependent mechanism of endothelial dysfunction of the penile vascular bed associated with atherosclerosis and hypercholesterolemia.

Numerous animal and human experimental data have demonstrated that diabetic vasculopathy and neuropathy contribute significantly to diabetic-associated ED. Impairments in endothelium-dependent and NANC-mediated cavernosal smooth muscle relaxation are well established in diabetic corpus cavernosum in vitro and in vivo (Saenz de Tejada et al, 1989; Vernet et al, 1995; Rehman et al, 1997; Way and Reid, 1999; Gur et al, 2000; Cartledge et al, 2001b; Bivalacqua et al, 2003b). Recent evidence suggests that oxidative stress may play a prominent role in diabetic endothelial dysfunction of the penile vascular bed. Endothelium-dependent cavernosal smooth muscle relaxation is impaired in organ bath studies with alloxan-induced diabetic rabbit cavernosal tissue (Khan et al, 2001). SOD treatment restored endothelial- and NANC-mediated corpus cavernosum smooth muscle relaxation, demonstrating the functional significance of superoxide anion in mediating diabetic endothelial dysfunction in vitro. Ryu et al (2003) have shown that malondialdehyde, a lipid peroxidation product that is a measure of oxidative stress, is increased in cavernosal tissue obtained from streptozotocin (STZ)-induced rats with diabetes. Our lab has recently found a significant up-regulation of superoxide anion, driven by the membrane-bound NADH/NADPH oxidase, in cavernosal tissue of STZ-induced rats with diabetes with no change in total SOD activity (Bivalacqua et al, unpublished data). These animals had concomitant ED as measured by cavernosal nerve-induced erectile responses in vivo. Adenoviral gene transfer of EC-SOD to the diabetic rat penis reduces the marked increase in cavernosal superoxide anion levels and completely restored cavernosal nerve-induced erectile function in this cohort of animals (Bivalacqua et al, unpublished data). These results suggest that an increase in superoxide anion formation caused by an up-regulation of NADPH oxidase without a compensatory increase in SOD occurs in the penis of the STZ-induced rat with diabetes and that gene therapy of EC-SOD can reduce superoxide anion levels and restore erectile function in vivo. These studies provide evidence that in diabetes superoxide radicals are increased, possibly by the up-regulation of NADPH oxidase, and contribute significantly to endothelial dysfunction of the penile vasculature in diabetes.

Aging is associated with marked changes in the penis, especially in the endothelium and smooth muscle cells (Harman, 2001; Drew and Leeuwenburgh, 2002). Oxidative stress is an important factor contributing to vascular dysfunction in aging (Hamilton et al, 2001; Taddei et al, 2001). Superoxide anion levels are increased in the cardiovascular system and a number of peripheral vascular beds with advancing age. Therefore, age-related endothelial dysfunction of the penis may involve mechanisms such as increased oxidative free radicals or alterations in antioxidant defense systems. Aging is associated with decreased NO-bioavailability and responsiveness to endothelium-dependent vasodilator stimuli in the corpus cavernosum, and the role superoxide anion plays in mediating this decreased responsiveness had not been established until recently. Because superoxide anion reacts with NO to form peroxynitrite, this reaction does not allow NO to perform its role in vasodilation and penile erection. In the aging penis, overproduction of toxic radical peroxynitrite could cause degeneration of nerves and endothelial cells involved in the erectile process (Ferrini et al, 2001; Bivalacqua et al, 2003a). Thus, reduced endothelial-derived NO may be a result of increased production of superoxide anion in the aged penis. Recently, in situ detection of superoxide was shown to be elevated in endothelial and cavernosal smooth muscle cells of the aged rat (Figure 5) (Bivalacqua et al, 2003a). Although there was an increase in superoxide anion formation, there was no change in total SOD activity, such that there was an imbalance in superoxide anion generation and inactivation in the penile vasculature of the aged rat. This increase in superoxide anion generation is associated with decreased NO synthesis in the penis, thus impairing endothelial-dependent smooth muscle relaxation and resulting in reduced erectile function (Bivalacqua et al, 2003a). It was this imbalance in superoxide anion formation and SOD activity that led us to hypothesize that increasing EC-SOD expression might be beneficial in limiting superoxide anion formation. In rats transfected with Ad-CMVEC-SOD, expression of EC-SOD mRNA and protein was significantly higher and resulted in a significant reduction in superoxide anion formation in the aged rat penis. Additionally, EC-SOD gene therapy reduced peroxynitrite formation and increased cGMP levels in the penile vasculature that was associated with enhanced in vivo erectile responses to cavernosal nerve stimulation and the endothelium-dependent vasodilator acetylcholine in the aged rat (Bivalacqua et al, 2003a). These data indicate a pathophysiologic role for superoxide anion in augmenting erectile function in aging by reducing bioavailability of NO and impairing endothelial function in the penis.

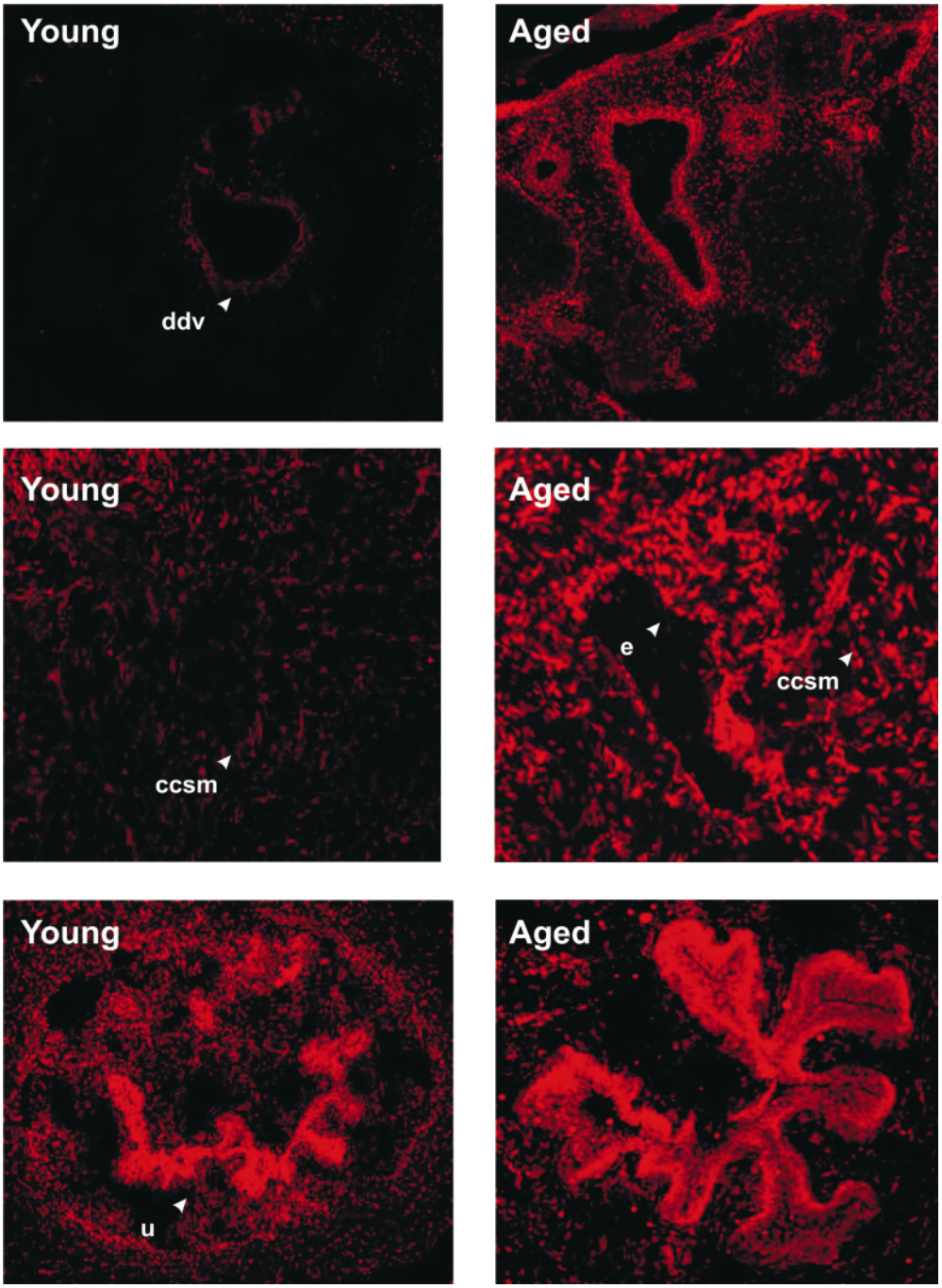

Figure 5. In situ detection of superoxide anion in young and aged rat penises. Confocal fluorescent photomicrographs of young and aged rat penises incubated with hydroethidine. Hydroethidine is freely permeable to cells and, in the presence of superoxide anion, is oxidized to red-fluorescent ethidium bromide (EtBr), where it is trapped by intercalation with DNA. This method provides sensitive detection of superoxide anion levels in situ. Penises of young rats demonstrate minimal fluorescence in the deep dorsal vein and small arterioles of the penis, endothelium, and corpus cavernosal smooth muscle. In aged rat penises, there is a marked increase in ethidium bromide fluorescence reflecting increased superoxide anion levels in the deep dorsal vein, small arterioles, endothelium, and corpus cavernosal smooth muscle. e indicates endothelium; ccsm, corpus cavernosum smooth muscle; ddv, deep dorsal vein; u, urethra.

Endothelial Dysfunction and Hypercholesterolemia in the Penis

Hypercholesterolemia and subsequent atherosclerosis are well-recognized risk factors for the development of vasculogenic ED (Sullivan et al, 2001). It is important to realize that hypercholesterolemia-associated endothelial dysfunction and subsequent ED in men are multi-factorial and are usually compounded by other vascular risk factors

such as smoking, age, and diabetes. The association of hypercholesterolemia and ED was originally attributed to atherosclerosis of the hypogastric cavernosal arterial bed that resulted in a reduction in arterial inflow to the penis (Azadzoi and Goldstein, 1992; Azadzoi et al, 1996). More recently, the importance of endothelium-dependent cavernosal smooth muscle relaxation has become apparent. Experimental hypercholesterolemia in animal models leads to a reduction in endothelial and cavernous smooth muscle cells with loss of intercellular contacts in the corpus cavernosum sinusoids as well as myelinated and nonmyelinated nerves of the penis (Yesilli et al, 2001; Gholami et al, 2003). Examination of the iliac arteries in hypercholesterolemic rabbits demonstrates significant atherosclerotic plaques and atherosclerotic-like processes in focal areas of the cavernosal sinusoids (Behr-Roussel et al, 2002). In animal models of hypercholesterolemia, in vitro impairments in endothelium-dependent relaxation to acetylcholine in the corpus cavernosum exist, whereas NANC-selective electrical field stimulation is unchanged and endothelium-independent relaxation to NO-donors may be preserved or reduced (Azadzoi and Saenz de Tejada, 1991; Behr-Roussel et al, 2002). Moreover, eNOS enzyme activity is reduced in cavernosal tissue obtained from rabbits with hypercholesterolemia, and nNOS enzyme activity is not altered (Seo et al, 1999). In rabbits with hypercholesterolemia, erectile function was reduced to pelvic nerve stimulation in vivo as well as significant impairments in endothelium-dependent and -independent relaxations of corpus cavernosum in vitro (Seo et al, 1999). These studies document significant impairments in endothelium-dependent smooth muscle relaxation and eNOS enzyme activity in the penis as a result of hypercholesterolemia, thus highlighting the importance of endothelial-derived NO in the maintenance of erectile function.

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen in vitro and an angiogenic growth factor in vivo (Kroll and Waltenberger, 1998; Six et al, 2002). VEGF upregulates eNOS in corpus cavernosal smooth muscle cells and elicits an increase in production of NO in human endothelial cells grown in culture (Lin et al, 2002). VEGF maintains penile erection in a mouse model of vasculogenic ED by phosphorylation/activation of Akt and eNOS, suggesting that eNOS mediates VEGF-promoted erection (Musicki et al, 2002). The use of VEGF gene therapy to improve endothelial cell function in the penis of hypercholesterolemic animals has been demonstrated. Both intravenous and intracavernous administration of VEGF increased endothelial cell content and improved endothelium-dependent smooth muscle relaxation of the corpus cavernosum (Henry et al, 2000; Byrne et al, 2001; Gholami et al, 2003). These studies demonstrate that VEGF can protect in vitro endothelium-dependent smooth muscle relaxation of the corpus cavernosum of hypercholesterolemic animals. Future studies are warranted to determine if VEGF gene therapy can reverse endothelial dysfunction of the penile vascular bed once atherosclerosis and hypercholesterolemia are present and if this therapy can improve erectile function in vivo.

Endothelial Dysfunction and Diabetes in the Penis

Peripheral vascular disease and neuropathy of autonomic nerves are established complications associated with diabetes mellitus. Such alterations in the peripheral vasculature may underlie the high prevalence (>50%) of diabetic ED in men (Hakim and Goldstein, 1996). A major factor contributing to diabetic ED in human corporal tissue is a reduction in the number of nitrergic NOS containing nerve fibers, constitutive NOS activity, and impaired endothelial- and neurogenic-mediated smooth muscle relaxation (Saenz de Tejada et al, 1989; Cellek et al, 1999; Bivalacqua et al, 2001b). Studies have demonstrated that animals with chemically induced and genetic diabetes have significant decreases in penile eNOS and nNOS protein/gene expression and cavernosal cGMP levels at a time when in vivo erectile function to cavernosal nerve stimulation and intracavernous acetylcholine, reflex erectile function, and mating behavior are impaired (Ari et al, 1999; El-Sakka et al, 1999; Akingba and Burnett, 2001; Podlasek et al, 2001; Thompson et al, 2001; Escrig et al, 2002; Bivalacqua et al, 2003b). However, other biochemical measurements have reported NOS activity to be increased, and autoradiographic localization revealed increased binding of eNOS in penile tissue of rats with diabetes, which may be a compensatory mechanism to increase NO biosynthesis (Sullivan et al, 1996, 2002). Interestingly, endothelium-independent cavernosal smooth muscle relaxation is also impaired in animal models of diabetes, suggesting that diabetes attenuates endothelial- and neurogenic- NO neurotransmission but may also affect smooth muscle reactivity and the downstream second messengers soluble guanylate cyclase, cGMP, or protein kinase cGKI (Way and Reid, 1999). Collectively, these studies document a probable mechanism for the development of ED in diabetes that is a dysfunctional regulation of the NO/cGMP system with a decrease in NO production via a reduction in both eNOS and nNOS in the penile vasculature.

Because NO-derived smooth muscle relaxation is impaired in diabetes, administration of L-arginine, the substrate for NOS, could improve NO biosynthesis and restore corporal smooth muscle relaxation. Long-term oral administration of L-arginine to rabbits with diabetes increased endothelium-dependent corporal smooth muscle relaxation, whereas L-arginine treatment did not restore impaired neurogenic relaxation, suggesting that L-argi-

nine transport or availability to interact with eNOS in the endothelium is reduced, thus contributing to endothelial dysfunction in these rabbits (Yildirim et al, 1999). The enzyme arginase competes with eNOS for the substrate L-arginine in endothelial cells throughout the vascular system. Therefore, increased expression of arginase in diabetic corpus cavernosum may reduce endothelial-derived NO biosynthesis by reducing eNOS activity. We recently demonstrated that arginase activity and arginase II protein and gene expression were significantly increased in diabetic human corpus cavernosum when compared with nondiabetic cavernosal tissue (Bivalacqua et al, 2001b). Additionally, diabetic cavernosal tissue had a significantly lower conversion of L-arginine to L-citrulline, suggesting that the calcium-dependent NO pathway by predominately eNOS is reduced in diabetic tissue. In the presence of ABH, the calcium-dependent conversion of L-arginine to L-citrulline was increased significantly in diabetic cavernosal tissue. These data may suggest that the elevated arginase expression/activity reduces eNOS activity by outcompeting the eNOS for L-arginine, and that inhibition of arginase by ABH shifts the availability of L-arginine to eNOS, thus resulting in increased conversion of the substrate to NO.

Because impairments in endothelial function dramatically affect erectile function in diabetes, in vivo gene transfer of eNOS could have beneficial physiological effects on penile erection in a condition that is associated with endothelial dysfunction and decreased eNOS expression. Therefore, our laboratory used the STZ rat with diabetes model to test the effect of adenoviral gene transfer of eNOS on diminished erectile responses found in this rat model (Bivalacqua and Hellstrom, 2001; Bivalacqua et al, 2003b, in press). We found that diabetes caused significant reduction in cavernosal eNOS protein, enzyme activity, cGMP levels, and significant reduction in erectile response to direct injection of acetylcholine and cavernosal nerve stimulation (Figure 6). After successful adenoviral gene transfer of eNOS to the diabetic rat penis, an increase in cavernosal eNOS protein, enzyme activity, and cGMP formation was present in the corpus cavernosum that resulted in a restoration of erectile function to cavernosal nerve stimulation and endothelium-dependent erectile responses to acetylcholine (Figure 6) (Bivalacqua et al, 2003b, in press). These results demonstrate that adenoviral-mediated gene transfer of eNOS to the diabetic corpus cavernosum corrects impaired endothelial-derived NO-mediated erectile response.

The interactions of endothelial-derived NO with the RhoA/Rho-kinase-signaling pathway have only recently been investigated. The potential role of RhoA in endothelial dysfunction arose from clinical studies with statins, which lower cholesterol and improve endothelial function by decreasing posttranslational acylation of RhoA GTPase and restore diminished eNOS protein and gene expression (Laufs and Liao, 1998). Direct inhibition of RhoA by the dominant negative RhoA mutant leads to increases in aortic eNOS expression and activity in mice (Laufs et al, 2000). Recent studies have demonstrated that RhoA/Rho-kinase pathways play an important role in suppression of eNOS gene expression and enzyme activity in human endothelial cells, which contributes to decreased endothelial-derived NO production, a hallmark characteristic of diabetic-associated vascular dysfunction (Ming et al, 2002). Therefore, this novel-signaling pathway may add to our understanding of the mechanisms of augmented endothelium-dependent smooth muscle relaxation in diabetic endothelial dysfunction.

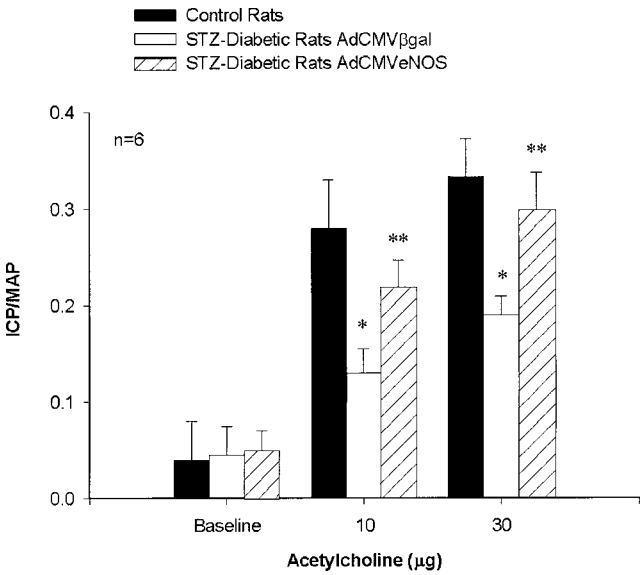

Figure 6. Bar graphs showing the increase in intracavernosal pressure (ICP/MAP) in response to direct intracavernosal injection of the endothelium-dependent vasodilator acetylcholine in age-matched control and streptozotocin (STZ) rats with diabetes transfected with AdCMVβgal or AdCMVeNOS. In vivo erection experiments were conducted 2 days after transfection with adenoviruses. STZ rats with diabetes had significant reductions in erectile response to the intracavernous injection of acetylcholine, suggesting these rats exhibited endothelial dysfunction of the penile vascular bed. After gene therapy with eNOS, endothelium-dependent erectile responses to acetylcholine were restored. n indicates number of experiments; *, response significantly different compared with control rats ($P < .05$); **, response significantly different compared with STZ rats with diabetes transfected with AdCMVβgal ($P < .05$).

The role of RhoA/Rho-kinase in the pathogenesis of diabetic-associated ED has not been fully delineated. Recently, Chang et al (2003) reported that Rho-kinase is increased in corpus cavernosum obtained from alloxan-induced rabbits with diabetes. STZ-induced rats with diabetes also have an increase in RhoA and Rho-kinase in diabetic corpus cavernosum at a time when eNOS protein and activity is reduced (Bivalacqua et al, 2003d). This increase in RhoA/Rho-kinase expression was associated with enhanced in vivo erectile responses to the Rho-ki-

nase inhibitor, Y-27632, but a decrease in erectile response to cavernosal nerve stimulation, suggesting that the diminished erectile response in the STZ rats with diabetes may be due to decreased endothelial-derived NO as a result of increased Rho-kinase expression as described in other peripheral vascular beds (Figure 4). In order to test this hypothesis, we directly injected into the penis of the STZ rat with diabetes an adeno-associated viral vector encoding for a dominant negative RhoA mutant to reduce RhoA/Rho-kinase expression in the rat's corpus cavernosum. After gene transfer of the dominant negative RhoA mutant, erectile responses to cavernosal nerve stimulation were similar to the age-matched controls, and RhoA/Rho-kinase expression was significantly reduced (Bivalacqua et al, 2003d). Moreover, eNOS protein and constitutive NOS activity were significantly increased with no change in nNOS protein expression. These data suggest that in experimental models of diabetes, there is an up-regulation of cavernosal Rho-kinase expression as a result of diminished endothelial-derived NO production in the penis. Collectively, these studies document a novel mechanism that may play an important role in the pathogenesis of endothelial and ED in diabetes.

Hyperglycemia is the defining characteristic of type 1 and 2 diabetes. Glucose is known to bind nonenzymatically to free amino acids on proteins or lipids. Through a series of oxidative and nonoxidative reactions, advanced glycation end products (AGEs) are formed irreversibly and accumulate in tissues over time, in particular endothelial and vascular smooth muscle cells (Singh et al, 2001). Although AGE formation occurs during the natural aging process, it is markedly increased in diabetes as a consequence of an increase in glucose (Jiaan et al, 1995). A common consequence of AGE formation is the pathologic cross-linking of collagen, which leads to vascular thickening with loss of elasticity, endothelial dysfunction, and ultimately atherosclerosis of the vascular tree.

AGEs are known to quench NO in vitro, and AGE formation is associated with accelerated superoxide anion formation (Vlassara, 2001). AGEs accumulate in endothelial and smooth muscle cells and cause sustained cellular activation of various proteins and generation of oxygen-derived free radicals. AGEs have been shown to affect eNOS by intracellular glycation of the enzyme at base pairs 599 to 602 and alteration of eNOS activity (Seftel et al, 1997). AGEs are increased in human diabetic cavernosal tissue when compared with controls, suggesting that AGE formation may be involved in the pathogenesis of diabetic endothelial and ED (Seftel et al, 1997). In animal models of diabetes, aminoguanidine (an inhibitor of AGEs) can improve in vitro endothelium-dependent cavernosal smooth muscle relaxation and in vivo erectile responses to cavernosal nerve stimulation by direct inhibition of AGE formation, down-regulation of its receptor galectin-3, and decreased collagen glycation in the STZ-diabetic penile vasculature (Cartledge et al, 2001a; Usta et al, in press). Moreover, aminoguanidine prevented the time-dependent progression of impaired erectile responses in STZ rats with diabetes (Usta et al, unpublished data). The effects of aminoguanidine on erectile physiology are difficult to interpret because this pharmacological compound is also an inhibitor of iNOS. Additionally, aminoguanidine may prevent diabetes-induced changes in the connective tissue composition of the microvascular wall of the arterioles supplying the penis, thus improving arterial inflow to the penis. Taken together, the deleterious of AGEs seem to be involved in the pathogenesis of endothelial dysfunction as it relates to diabetes.

Endothelial Dysfunction and Aging in the Penis

The aging penile vascular tree undergoes characteristic changes involving the arterial and vascular beds that include endothelial dysfunction. The mechanisms underlying aged-related vascular endothelial dysfunction likely involve multiple signaling pathways; however, the hallmarks of aged-associated ED is an elevated vasoconstrictor tone and decreased endothelium- and neurogenic-mediated relaxation of the penile vascular tree. Aging is recognized to alter endothelial cell function, and the decrease in aged-related erectile function (cavernosal nerve stimulation and intracavernous erectile response to acetylcholine) has been attributed to reductions in NANC nerve fibers in the penis, decreased constitutive NOS activity, impaired endothelial-dependent smooth muscle relaxation, diminished NO-bioavailability, and increased degradation or scavenging of NO (Garban et al, 1995; Carrier et al, 1997; Bivalacqua et al, 2000b, 2003a; Cartledge et al, 2001b). The endothelial dysfunction observed in the aging penis is not necessarily due to a decrease in eNOS protein. Normal and elevated levels of eNOS protein have been reported in the corpus cavernosum from a number of different animal species (Haas et al, 1998). The elevation of eNOS protein in the aged corpus cavernosum is likely a result of reduced expression of caveolin-1 and increased ROS, which in turn favors uncoupling of eNOS and increased expression of a dysfunctional enzyme (Bakircioglu et al, 2001; Bivalacqua et al, 2003a). However, eNOS activity and endothelial derived NO biosynthesis are reduced in the aged (more than 18 months) rat and mouse penises (Figure 7) (Bivalacqua et al, 2002). Therefore, endothelial dysfunction and reduced NO biosynthesis in the aged penis are in part mediated by an increase in production of ROS and reduced enzyme activity of eNOS in the penile vasculature.

A reduction in nNOS nerve fibers has been demonstrated in the aged rat penis (Carrier et al, 1997). This reduction in NANC nerves is thought to contribute to diminish

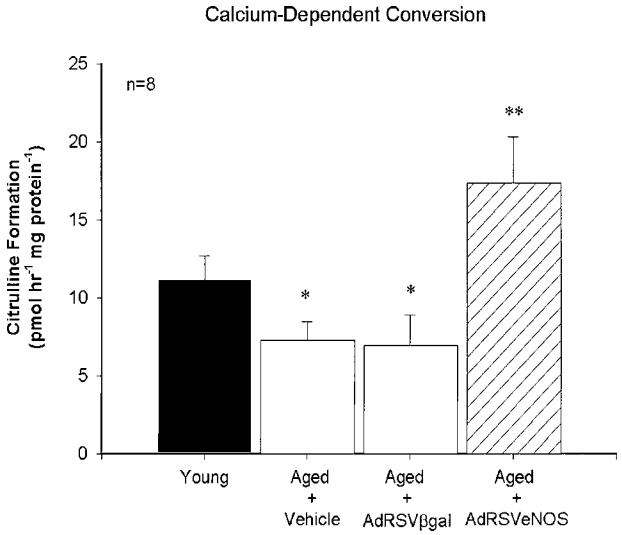

Figure 7. NOS activity as measured by calcium-dependent conversion of L-arginine to L-citrulline in young (3 months) and aged (18 months) rat cavernosal tissue 7 days after transfection with vehicle, AdRSVβgal, and AdRSVeNOS. Aged cavernosal tissue had a significant reduction in constitutive NOS activity, which is primarily a measure of eNOS activity. After gene therapy with eNOS, constitutive NOS activity was restored to levels greater than those found in young rats. *n* indicates number of tissue samples; *, $P < .05$ when compared with young; **, $P < .05$ when compared with aged cavernosal tissue transfected with vehicle or AdRSVβgal.

erectile response to cavernosal nerve stimulation and reduce penile reflexes in rats. However, the independent role of eNOS and its capacity to improve endothelial function and erectile function in aging was unknown until recently. Using novel gene transfer technologies, our laboratory was able to deliver an adenovirus that encoded the eNOS isoform to the penis of aged rats. In vivo adenoviral gene transfer of eNOS resulted in an elevation of eNOS protein, mRNA, constitutive NOS activity (Figure 7), endothelial-derived NO, and cavernosal cGMP levels (Champion et al, 1999; Bivalacqua et al, 2000b). There was no change in nNOS protein expression. The end result of overexpression of eNOS in the aged rat penis was a physiologically relevant change in erectile function. There was both an increase in erectile function as measured by cavernosal nerve stimulation and an improvement in endothelial function in that intracavernous Ach erectile responses were significantly improved after eNOS gene therapy. These data establish that the eNOS transgene has biological activity in the rat penis and can reverse the age-related endothelial dysfunction and ED associated with a reduction of NO biosynthesis from the vascular endothelium of the penis.

Another possible explanation for the elevated levels of eNOS protein but reduction in constitutive NOS activity and NO production in the aged rat penis may be a problem with L-arginine availability for eNOS. The enzyme arginase can reciprocally regulate eNOS activity in the vascular endothelium; therefore, if arginase is increased in aging, it may also be involved in the pathophysiology of age-related reductions in endothelial derived NO in the penis (Mori and Gotoh, 2000). In order to test this hypothesis, we isolated endothelial cells from young and aged mice penises and measured arginase I expression and activity. We found that both arginase activity and arginase I mRNA and protein expression are increased in aged cavernosal endothelial cells in vitro as well as whole corpus cavernosum from the mice (Bivalacqua et al, 2002). Using pharmacological inhibitors of arginase and an adeno-associated virus encoding for an antisense sequence to arginase I, we found that by inhibiting arginase expression and activity in vitro in endothelial cells we could restore eNOS activity (Bivalacqua et al, 2002). More importantly, adeno-associated viral gene transfer of the antisense for arginase I restored endothelium-dependent erectile responses to Ach and cavernosal nerve stimulated erectile response through inhibiting arginase I expression and restoring eNOS enzyme activity in the aged mouse penis (Bivalacqua et al, 2002). This study suggests that with advancing age, expression and function of arginase increases in the penile vascular bed and contributes to endothelial dysfunction and ED as it relates to aging. These data provide evidence that arginase may be a valuable target, especially as it relates to aging, in the treatment of ED in disorders with reduced eNOS activity and NO biosynthesis.

Endothelial Dysfunction and Endogenous NOS Inhibitors

Analogs of L-arginine are capable of NOS inhibition, thereby reducing its availability to interact with L-arginine and thus decreasing NO biosynthesis. An endogenous compound called asymmetric dimethylarginine (ADMA, or N^G,N^G-dimethyl-L-arginine) has been shown to competitively inhibit eNOS, thereby reducing endothelial-derived NO and endothelium-dependent vasodilation. The source of ADMA within endothelial cells is formed by the catabolism of proteins containing methylated arginine residues. The enzyme *S*-adenosylmethionine methylates internal arginine residues in a variety of polypeptides, yielding N^G-monomethyl-L-arginine (L-NMMA), ADMA, and symmetric dimethylarginine (SDMA or N^G,$N^{G''}$-dimethyl-L-arginine) upon proteolysis. These endogenous NOS inhibitors, especially ADMA, are associated with dysfunction of the vascular endothelium in a number of organic vascular diseases (Boger and Bode-Boger, 2000). Healthy patients have plasma levels of ADMA near 1 μmol/L, but in individuals with hypercholesterolemia ADMA levels are elevated to 2.2 μmol/L (Boger et al, 2001). Additionally, increased ADMA levels are associated with impaired endothelium-dependent forearm vasodilation in patients with hypercholesterolemia,

suggesting that this endogenous NOS inhibitor impairs endothelial function (Boger et al, 2001). Intra-arterial infusion of ADMA in health volunteers caused significant reductions in forearm blood flow (Vallance et al, 1992). Recently, plasma ADMA levels have been shown to be increased, independent of other risk factors, in patients with coronary artery disease and diabetes (Walker et al, 2001). These studies suggest that ADMA may be a marker of endothelial dysfunction as related to cardiovascular risk factors but may also cause endothelial dysfunction associated with various vascular diseases.

Evidence for the involvement of ADMA or other endogenous NOS inhibitors in the pathogenesis of endothelial dysfunction as it relates to ED have only recently been evaluated. A rabbit model of cavernosal ischemia that impaired endothelium-dependent vasodilation in isolated corpus cavernosum after ischemia was associated with decreased NOS activity and increased accumulation of L-NMMA and ADMA (Masuda et al, 2002). Excess administration of L-arginine in this model partially restored endothelium-dependent relaxations. Future studies in conditions associated with endothelial dysfunction and ED (eg, hypercholesterolemia, diabetes, atheroscelerosis, renal disease, aging) will need to be conducted in order to determine an association of elevated levels of endogenous NOS inhibitors and impaired cavernosal endothelial cell function in the penis.

Conclusion

The endothelium plays a vital role in maintaining vascular homeostasis in the penis. Endothelial-derived NO plays an integral role in the physiology of erection both in normal and in pathological disease states. The earliest detectable changes in vascular disease states associated with ED are abnormalities of the endothelium, causing a loss in its normal homeostatic mechanisms that conventionally protect against disease-related processes. Given the central importance of the endothelium, it is not surprising that conditions that cause endothelial dysfunction, such as aging, diabetes, cardiovascular disease, and hypercholesterolemia, are closely associated with ED. Indeed, normal penile erection requires coordinated arterial endothelium-dependent vasodilation and sinusoidal endothelium-dependent corporal smooth muscle relaxation. Further discovery of the pathophysiological mechanisms involved in endothelial dysfunction as related to vascular diseases of the penis will undoubtedly lead to prevention strategies and endothelial-cell–based pharmacological or gene therapies for ED.

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