NEW APPROACHES FOR TREATMENT OF ERECTILE DYSFUNCTION IN
CONDITIONS OF LOW NITRIC OXIDE FORMATION: INVESTIGATION OF
RHO-KINASE INHIBITORS AND SOLUBLE GUANYLATE CYCLASE-
TARGETED THERAPIES

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I.   Prevalence of erectile dysfunction

Erectile dysfunction (ED) is a common and multi-factorial disorder in which

an individual cannot achieve or maintain a penile erection for adequate sexual

relations (1). Although ED is classified as a benign condition, it can have drastic

effects on the quality of life and self-esteem of men and their sexual partners.

ED is age-associated with 39% prevalence in men 40 years old and 67%

prevalence in men 70 years of age (1-3). Results from the Massachusetts Male

Aging Study (MMAS) reported that ED affects half of the general male population

between 40 and 70 years of age, with up to 600,000 new cases occurring in the

United States annually (2). Data from the ENIGMA study in 2004 suggested that

the condition occurs in approximately 17% of all European men (4). ED was

estimated to affect 150 million individuals in 1999 and it has been projected that

322 million men will suffer from the sexual disorder by the year 2025 (5).

ED (or impotence) was historically assumed to be a psychosomatic

disorder, however, the last 25 years have witnessed the advent of new and

effective pharmacotherapies for the condition that demonstrate the sexual

dysfunction has an organic etiology in most cases. ED is now considered a

comorbid disorder that presents with hypertension, diabetes mellitus,

atherosclerosis or Peyronie's disease and can be ascribed to biochemical,

biomechanical and/or structural abnormalities of the penis (6-9). Pelvic surgery

(including radical prostatectomy and radical cystectomy), illicit drug use,

alcoholism and use of pharmacologic agents such as β-blockers, diuretics and

antidepressants (e.g. selective serotonin reuptake inhibitors and serotonin &

norepinephrine reuptake inhibitors) have also been reported to induce ED (10-

14).

II.   Penile anatomy and histology

The penis is composed of the corpus spongiosum and the paired corpora

cavernosa (figure 1). The spongiosum is positioned ventrally along the penis and

serves as physiological support surrounding the urethra. The corpora

spongiosum also forms the glans penis distally. The glans penis is unique in that

it is covered with very thin, adherent skin containing fibrous connective tissue but

no fibrous sheath. The corpora cavernosa are the twin tissues that fill and

provide rigidity to the penis along the dorsal side of the organ. The corpora

cavernosa are each surrounded by the tunica albuginea, a bilayered structure

consisting of mostly collagen fibers, with some elastic fibers interspersed within
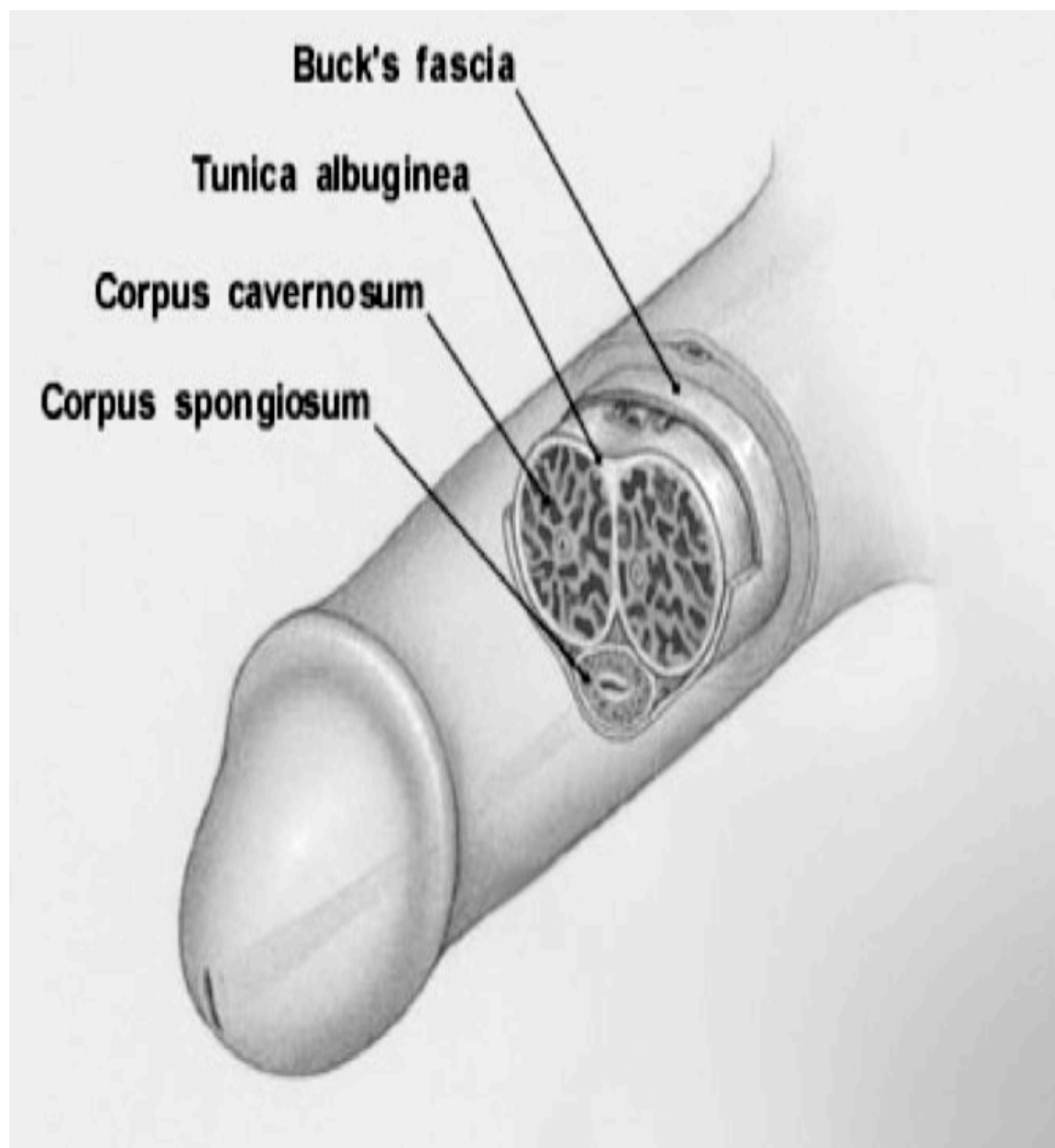
Figure 1. Cross section of male penis demonstrating three corporal bodies: paired corpora cavernosa and corpus spongiosum. Adapted from Fitkin and Ho, Am Fam Physician 1999 (15).

the sheath as well (16). In humans, the corpora cavernosa share an incomplete

perforated septum that allows them to physiologically act as a single functional

unit. The two corpora cavernosa as well as the corpora spongiosum are

surrounded by Buck's fascia, which is a dense fibrous structure from which

attached septa extend between the three corporal bodies (Fig. 1).

The primary arterial supply of the penis is the internal pudendal artery,

which is derived from the internal iliac artery and becomes the penile artery after

branching off the perineal artery in Alcock's canal. The penile artery supplies the

bulbar, urethral, dorsal and cavernous arteries. The cavernous artery travels

through the tunica albuginea to the proximal base, or crus, of both corpora

cavernosa and begins a series of multiple, corkscrew shaped arteries known as

the terminal helicine arteries that are 150-300 µm in diameter (17). These

helicine arteries act as resistance vessels and are responsible for filling the

cavernosal sinuses to initiate an erection (figure 2). The dorsal artery runs

underneath Buck's fascia in a bundle between the dorsal vein and dorsal nerves

and is responsible for engorgement of the glans penis during erection.

The venous system draining the penis is categorized based on anatomic

location. The deep venous system drains the three corporal bodies (paired

cavernosa and spongiosum) through the tunica albuginea with emissary veins.
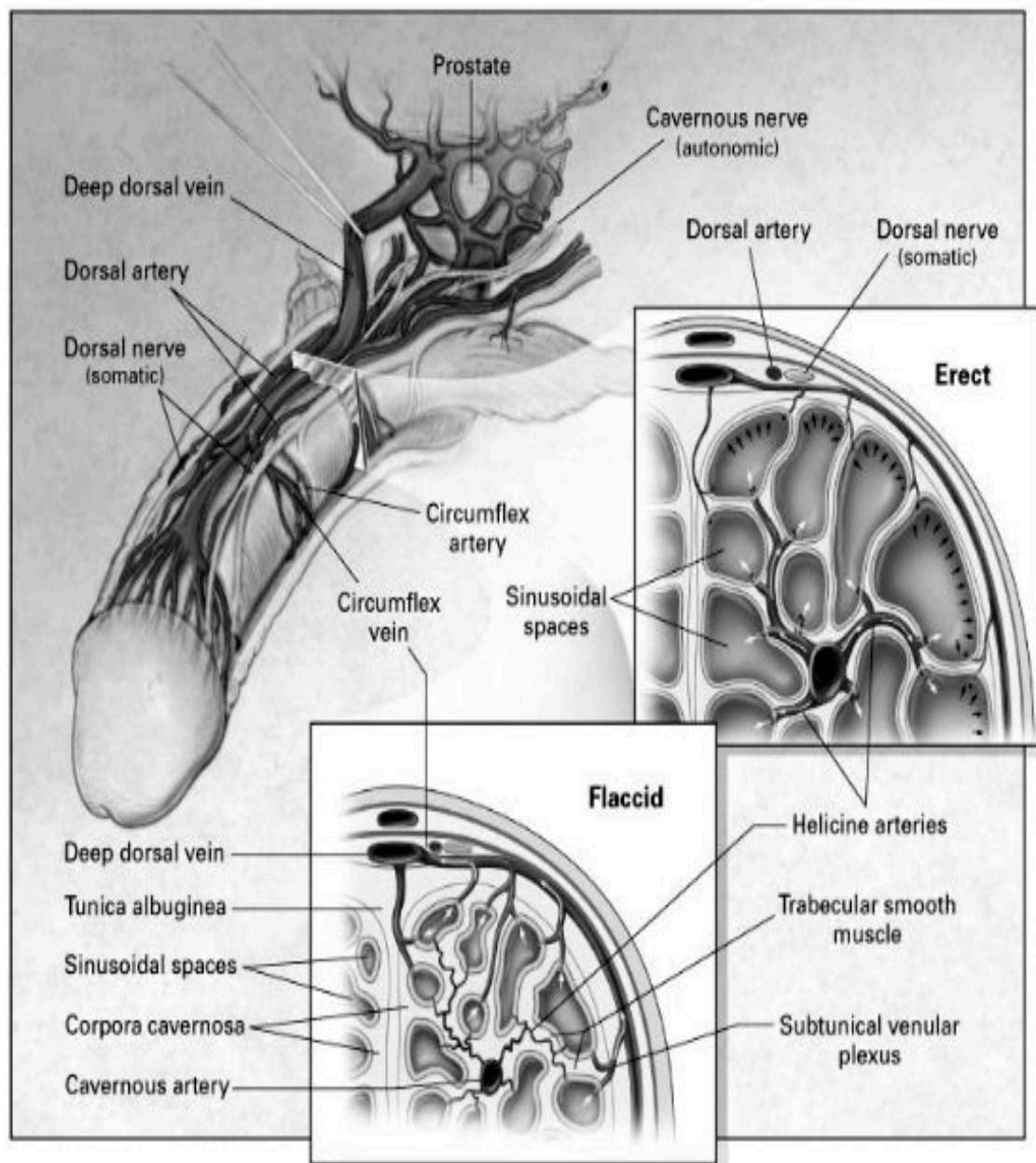
Figure 2. Vascular and neurologic system of penis in flaccid and erect state. Adapted from Lue, N Engl J Med 2000 (10).

The intermediate venous system lies outside of the tunica albuginea and underneath Buck's fascia. This vasculature is responsible for facilitating blood drainage within the glans penis. The superficial venous system is responsible for draining the skin and the tissue outside of Buck's fascia.

The tissue of the corpora cavernosa is composed of a meshwork of interconnected sinuses lined by vascular endothelium. Trabeculae consisting of smooth muscle cells (connected by gap junctions) surrounded by collagen, elastin and fibroblasts separate this "sponge-like" cavernosal tissue. Relaxation and constriction of smooth muscle lining the cavernosal sinuses and penile vasculature plays a large role in penile tumescence and flaccidity. When the penis is flaccid cavernous smooth muscle and the smooth muscle of penile arteries is predominantly contracted to allow minimal arterial inflow for nutritional purposes to tissues (17). Smooth muscle tone is proportional to the level of free cytosolic calcium (Ca^{2+}) within the cell. Norepinephrine released from nerve endings, endothelins and prostaglandin $F_{2\alpha}$ released from endothelial cells all activate receptors on smooth muscle cells to increase intracellular levels of inositol triphosphate and diacylglycerol via a phospholipase C-mediated pathway (18, 19). The accumulation of these intracellular messengers facilitates a release of Ca^{2+} from intracellular stores and opening of Ca^{2+} channels on the cell membrane. The increase of intracellular Ca^{2+} concentration results in a Ca^{2+}-calmodulin interaction with myosin light chain kinase (20). Phosphorylated

myosin light chains trigger cycling of myosin crossbridges along actin filaments and generation of force, as well as activation of a myosin ATPase that hydrolyzes ATP to provide necessary energy for contraction. A Ca^{2+} sensitization pathway also plays a role to regulate smooth muscle contraction with RhoA and Rho-kinase.

RhoA is a small, monomeric guanosine triphosphatase that regulates many physiologic processes including cellular growth and proliferation, immune responses and vascular smooth muscle contraction (21). Rho-associated protein kinases are downstream effectors for RhoA, and their activity increases the Ca^{2+} sensitivity of vascular smooth muscle. Rho-kinase is a serine/threonine kinase that phosphorylates and inhibits the regulatory subunit of myosin phosphatase within smooth muscle cells (22). This action maintains phosphorylation of myosin light chains and contractile activity within the smooth muscle (23). Interestingly, in 2002 Wang *et al.* reported that RhoA was found at a 17-times higher concentration in the penile smooth muscle when compared to ileal vascular smooth muscle in the rabbit (24).

III.   Physiology of the erectile response

Penile erection is an integrated, neurovascular phenomenon that combines central nervous system stimulation with peripheral release of

vasodilators from nerves and the endothelium of cavernosal tissues (17). The

central induction of the erectile response has been classified into three distinct

physiological processes: 1) reflexogenic induction, 2) psychogenic induction and

3) nocturnal penile erection. Nocturnal penile erection occurs during rapid eye

movement (REM) sleep, involves central nervous system activity and results from

a release of neurotransmitters and neuromodulators (serotonin, dopamine,

norepinephrine, glutamate, γ-aminobutyric acid and nitric oxide (NO)) that induce

their effects in the pontine reticular formation and the amygdalae (25, 26). A

psychogenic erection is triggered in response to external erotic sensory input or

can be fantasy-based. This stimuli is processed in the medial preoptic area

(MPOA) of the hypothalamus (27). Subsequently, the hypothalamus releases

several hormones such as gonadotropin releasing hormone, oxytocin, α-

melanocyte stimulating hormone and Substance P to enhance and propagate

central signaling for induction of an erection. In response to these hypothalamic

hormones, the activity of T_{11}-L_2 thoracolumbar sympathetic nerve fibers, which

normally function to inhibit the manifestation of an erectile response, is

attenuated. Concomitantly, these hormones also initiate neurotransmission of

sacral S_2-S_4 parasympathetic nerve fibers through the pelvic plexus and

cavernosal nerves. This parasympathetic activity results in the release of NO and

acetylcholine (ACh) from cavernosal nerve terminals to induce smooth muscle

relaxation and generate an erectile response. A reflexogenic erection can be

initiated through the same S_2-S_4 parasympathetic spinal centers via the sacral spinal reflex pathway by tactile stimulation of genital organs (28).

The collaborative efforts of Drs. Louis Ignarro and Jacob Rajfer at the UCLA Geffen School of Medicine identified NO as the principal peripheral mediator of erection in electrical field stimulation (EFS) studies with rodent and human cavernosal tissue (29, 30). The hypothesis about the role of NO in erection was further expanded in a 1992 *Science* publication in which Burnett *et al.*, demonstrated the pelvic ganglion of a rat and its surrounding nerves stained positive for the NO generating enzyme, nitric oxide synthase (NOS) (31). Activated NOS isoforms convert L-arginine and oxygen to L-citrulline and vasoactive NO utilizing nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme and tetrahydrobiopterin (BH_4) as cofactors for the reaction (32). Burnett's initial study on the role of NO in erection also demonstrated that erectile responses to electrical stimulation of the cavernosal nerve were decreased significantly after treating rats with the NOS inhibitor N-$^{\omega}$-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (31).

The cavernosal nerve innervating the penis consists of an adrenergic component, a cholinergic component, and a nonadrenergic, noncholinergic (NANC) component (Fig. 3) (10). The adrenergic component of the cavernosal

nerve functions to maintain flaccidity of the penis under normal conditions through release of norepinephrine. Released norepinephrine causes constriction of penile smooth muscle in conjunction with the Ca^{2+}-sensitizing mechanism of RhoA and rho-kinase (19). With sexual stimulation, the cholinergic component of the cavernosal nerve releases the neurotransmitter ACh and induces vasodilation by two different mechanisms: 1) ACh binds M3 muscarinic receptors on the cavernosal endothelium causing a transient increase in intracellular Ca^{2+} and activation of endothelial NOS (eNOS/NOS3) and 2) ACh binds muscarinic receptors on adrenergic nerve terminals resulting in an inhibition of the release of norepinephrine from the cavernosal nerve (27, 33). NANC neurotransmission occurs with sexual stimulation via an influx of Ca^{2+} through voltage-dependent N-type Ca^{2+} channels that occurs when action potentials reach the cavernosal nerve terminal. The increase in intracellular Ca^{2+} results in a Ca^{2+}-calmodulin interaction, which activates neuronal NOS (nNOS/NOS1) to generate NO from the substrate, L-arginine (Fig. 3). Similarly, when the M3 muscarinic receptors on penile endothelium bind ACh, the transient increase of Ca^{2+} that occurs in the endothelial cell in response to opening of ion channels results in Ca^{2+} binding calmodulin and subsequent NO production through activation of eNOS. These biochemical mechanisms demonstrate that both the eNOS and nNOS enzymes are activated through a Ca^{2+}-calmodulin interaction (34, 35). Recent studies have shown that each of the separate neurons of the cavernosal nerve may contain more than one neurotransmitter. ACh may be co-localized with nNOS,
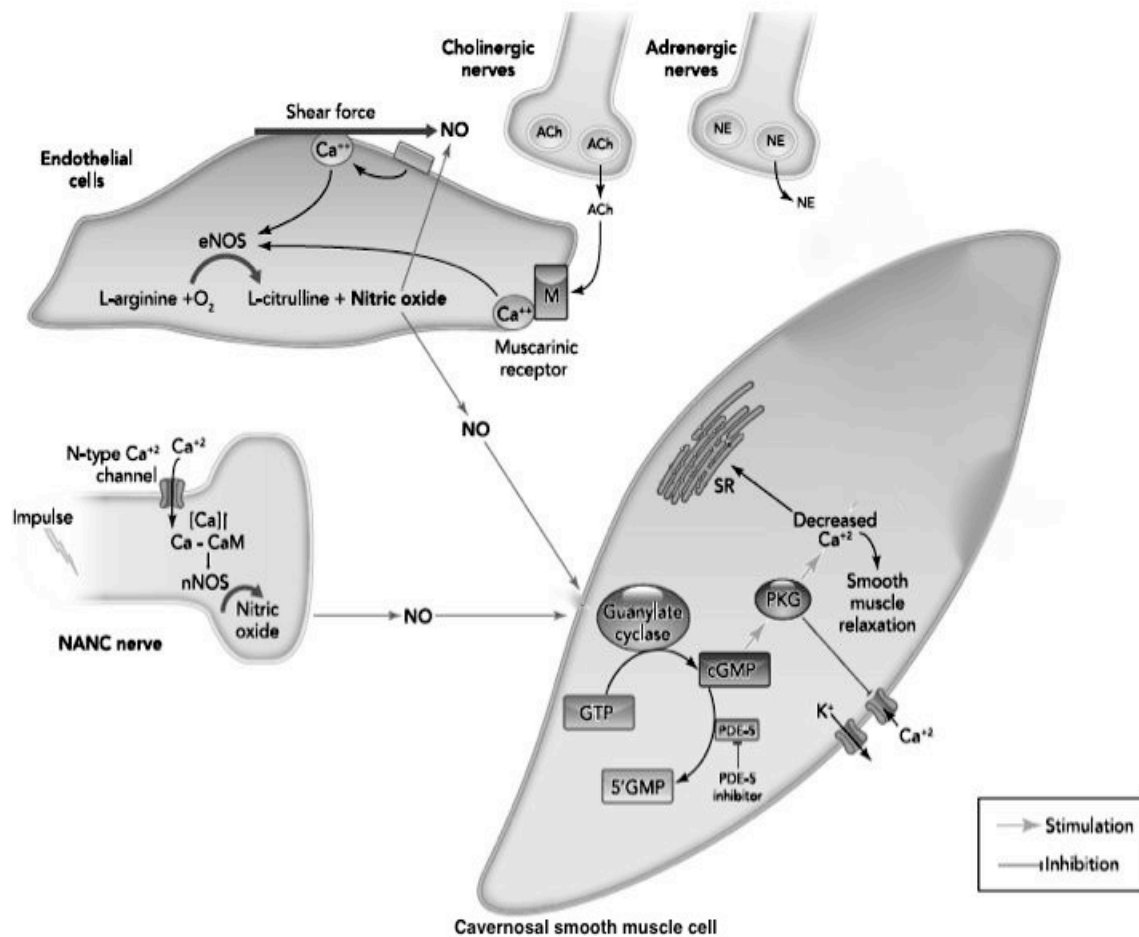
Figure 3. Diagram illustrating the three components (adrenergic, cholinergic and nonadrenergic, noncholinergic) that constitute the cavernosal nerve and major signaling pathways facilitating smooth muscle relaxation and penile erection. Adapted from Lasker *et al.*, Physiology (Bethesda) 2013 (36).

NO, vasoactive intestinal peptide, and neuropeptide Y in cholinergic nerve terminals, whereas NANC nerves have been shown to contain nNOS and enzymes such as the heme oxygenases (37-39).

The contribution of the cholinergic component of the cavernosal nerve to the erectile response is not well understood and remains controversial. Cholinergic receptors have been detected using radio-ligand binding studies in the cavernosal tissue of both humans and animals (17, 40-42). Studies on the effect of the cholinergic receptor antagonist atropine on erectile responses to cavernosal nerve stimulation experiments vary from complete blockade of the erectile response to no effect (figure 4) (43-50). It has been reported that intravenous (IV) injection of atropine in a dose of 1 mg/kg had no significant effect on the erectile response to pelvic nerve stimulation in the anesthetized dog (44). Dail and colleagues reported a similar observation in a rat model with the administration of atropine and stimulation of the pelvic nerves (47). Conversely, an early study in primates demonstrated that erectile responses to intracavernosal injection of ACh and cavernosal nerve stimulation were both significantly reduced, but not abolished, after administration of atropine (45). Shortly thereafter, the same group reported that erectile responses in dogs to intracavernosal injection of ACh were completely abolished after administration of 0.1 mg of atropine and the response to cavernosal nerve stimulation was decreased significantly after intracavernosal administration of the same dose
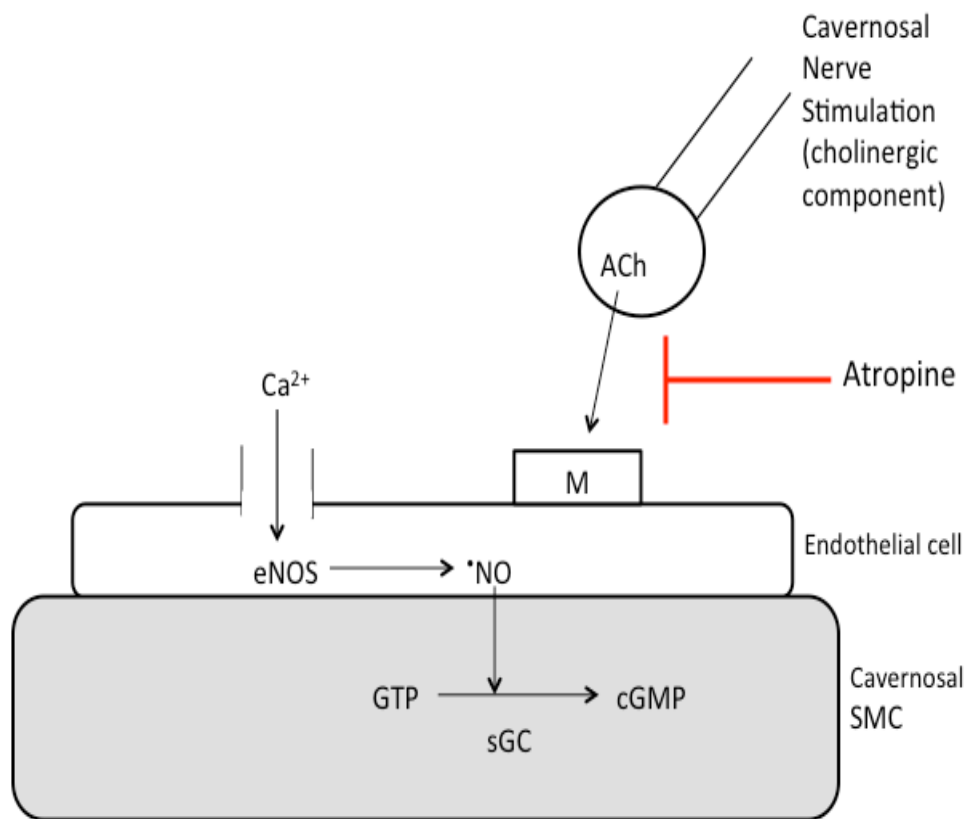
Figure 4. Attenuation of cholinergic signaling in penile endothelium and cavernosal smooth muscle by the competitive muscarinic receptor antagonist, atropine.

of the muscarinic antagonist (51). The role of muscarinic receptor activation

needs clarification in terms of its total contribution to NO-mediated erectile

responses.

As NO is released from the nerve terminals and the endothelium of the

corpora cavernosa, it diffuses into penile smooth muscle and binds to the

reduced iron (Fe^{2+}) on the beta-subunit of soluble guanylate cyclase (sGC),

increasing the catalytic activity of the heterodimeric enzyme (Fig. 5). Activated

sGC converts intracellular guanosine triphosphate (GTP) to cyclic guanosine

monophosphate (cGMP). Increased concentration of cGMP in smooth muscle

decreases bioavailable Ca^{2+} through activation of cGMP-activated protein

kinases (cGKs). cGK-1 activity induces opening of Ca^{2+}-gated potassium

channels which result in membrane hyperpolarization. Ca^{2+} influx through smooth

muscle membrane L-type channels is reduced and additional Ca^{2+} is taken up

into intracellular store (i.e. the sarcoplasmic reticulum) (52). This decrease in

bioavailable Ca^{2+} causes relaxation of smooth muscle in penile tissue. The

smooth muscle relaxation causes dilation of penile arteries and arterioles

resulting in increased blood flow into corporal sinuses in both cardiac systole and

diastole (11). The cavernosal sinuses expand while trapping arterial blood inflow.

Additional NO is released simultaneously through an eNOS-mediated

mechanism in response to the shear forces of increased blood flow along the

penile endothelium. With increased arterial inflow and hemodynamic engorgement of the organ, a compression of the subtunical venous plexuses between the tunica albuginea and the peripheral sinusoids takes place that reduces venous outflow from the penis. The tunica stretches to capacity and further occludes emissary veins between the inner circular and longitudinal layers further decreasing vascular outflow. Additionally, there is a myogenic response that occurs in the veins. The partial pressure of oxygen increases and the increase in intracavernosal pressure (ICP) raises the penis from a flaccid non-erectile state to a fully erect state (53).

Detumescence, or the cessation of an erection, involves several mechanisms including smooth muscle phosphodiesterase activity and vasoconstriction. The most important detumescence mechanism occurs through activation of a type 5 phosphodiesterase (PDE-5), which hydrolyzes cGMP to 5'-GMP (17). When cGMP levels decrease sufficiently in response to the PDE-5-mediated hydrolysis, cGK-1 activity ceases, intracellular Ca^{2+} levels increase, penile smooth muscle responds with contraction and the penis resumes a flaccid state.
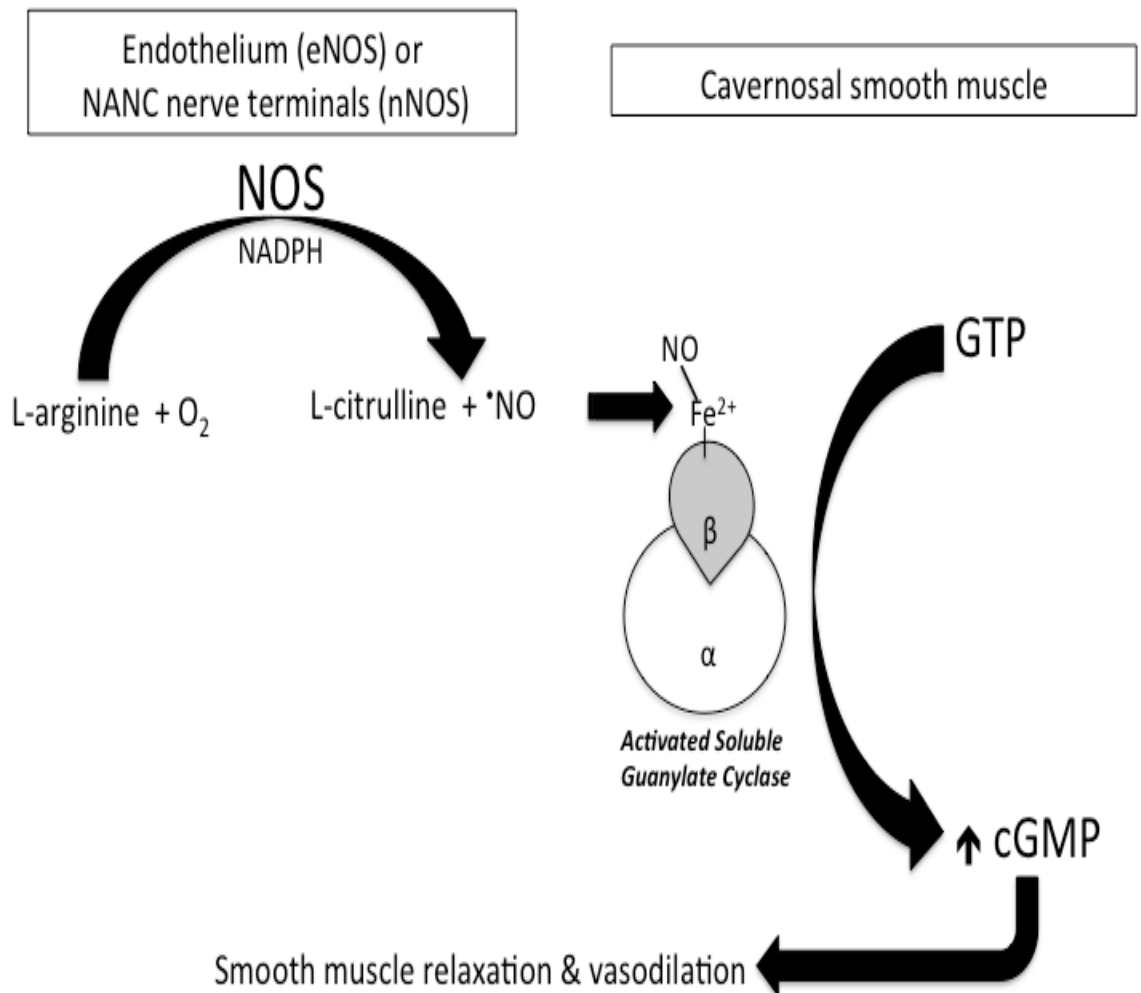
Figure 5. Soluble guanylate cyclase-mediated signaling in penile smooth muscle.

IV.   Pathophysiology of erectile dysfunction

Penile erection is dependent on sufficient synthesis and release of NO from the nerves innervating the corpora cavernosa and the endothelium lining penile vascular tissues. Therefore, a significant correlation exists between ED and pathophysiologic conditions involving decreased NO formation and/or bioavailability. In disease states such as heart failure, hypertension and diabetes mellitus, and in patients who have undergone pelvic surgery for prostate cancer, ED can occur (2, 11, 54, 55).

Reactive oxygen species (ROS) are endogenous products of oxygen metabolism such as hydroxyl radical (OH^-) and superoxide anion (O_2^-) that are generated in cells (56). Under normal conditions, the body has enzymatic antioxidant mechanisms in place including superoxide dismutase (SOD), catalase and other enzymes that function to scavenge oxygen radicals. However, under pathologic conditions ROS generation can be significantly increased resulting in oxidative damage to DNA, proteins, carbohydrates and lipids (56). ROS can also interact with NO to generate peroxynitrite ($ONOO^-$), which has been shown to reduce NO bioavailability (57). Peroxynitrite itself is not a free radical like NO and O_2^-, however, the molecule acts as a ROS that can induce further oxidative stress and cytotoxic effects (56, 57). It has been reported that O_2^- reacts with NO at a rate 3-fold faster than the rate at which the free radical

interacts with SOD, indicating a basal "quenching" of bioavailable NO under normal conditions by O_2^- (58). In conditions of increased oxidative stress, a significant reduction of NO bioavailability is kinetically favorable when compared to the activity of endogenous antioxidant defense mechanisms (56). Enhanced oxidative stress can also oxidize the heme moiety on the beta-subunit of sGC from a ferrous (Fe^{2+}) to a ferric (Fe^{3+}) state, which results in decreased capability of the enzyme to bind NO and induce cGMP-mediated vasodilation (figure 6). Therefore, oxidative stress can decrease erectile function and induce ED through: 1) generation of ROSs and 2) alteration of the redox state on the beta-subunit of sGC (36, 59).

Cardiovascular diseases and ED are closely related because both disease states involve impaired vascular endothelial function and a decreased bioavailability of endogenous NO. Therefore, a high co-prevalence between ED and cardiovascular disease exists. Risk factors such as hypertension, hypercholesterolemia, smoking, and diabetes mellitus are also common between the two disease states (60). An increase of ROS and prolonged oxidative stress have been implicated in the reduction of NO bioavailability associated with cardiovascular diseases (56). It can be assumed that micro-vascular diseases such as ED should precede macro-vasculopathies and studies have confirmed that ED is significantly associated with cardiovascular disease as well as cardiovascular disease mortality (61, 62).
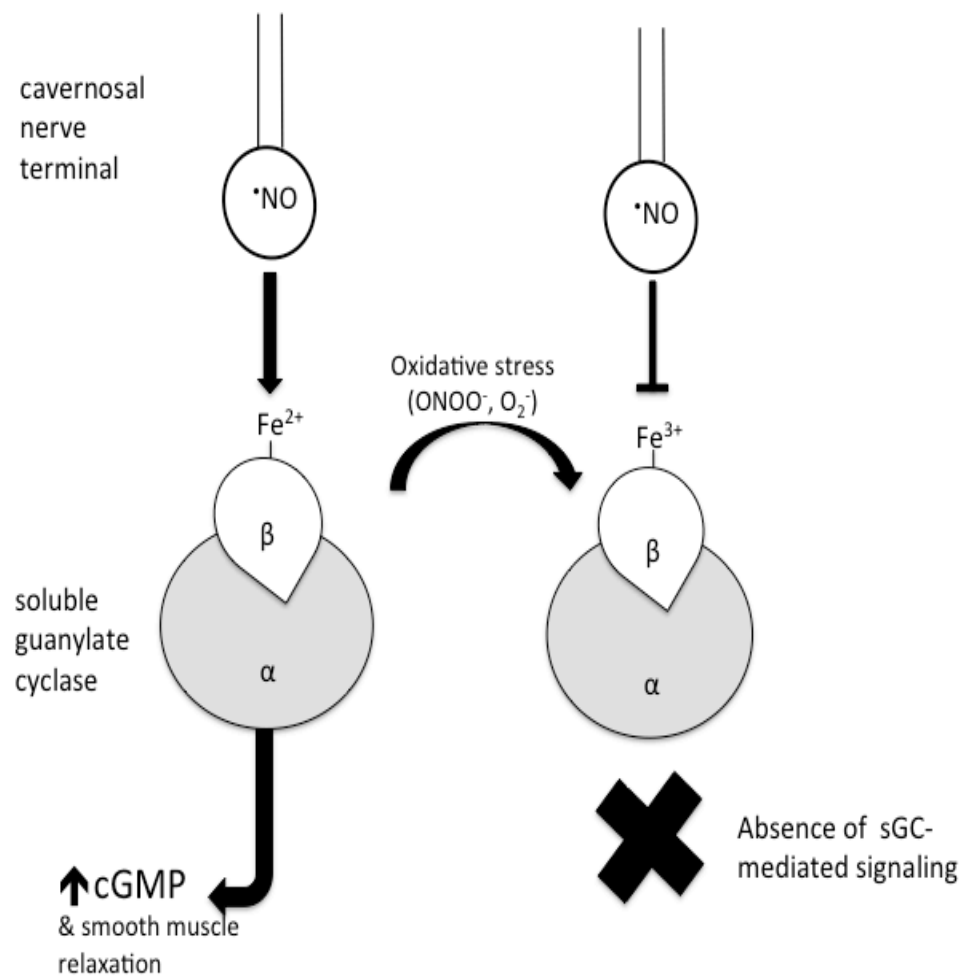
Figure 6. Prolonged oxidative stress that oxidizes the heme moiety on the beta subunit of soluble guanylate cyclase renders the intracellular receptor for nitric oxide incapable of activation and subsequent vasodilatory signaling through generation of cGMP.

In men with hypertension, arterial sclerosis, rather than high blood pressure, is associated with the development of ED (10). It has been shown in spontaneously hypertensive rats that corporal smooth muscle relaxation responses are inhibited before systemic vascular pathological changes occur (63). This suggests that changes in the endothelium associated with ED may precede systemic vascular dysfunction in hypertensive patients. Oxidative damage from increased O_2^- formation may also be important in the association between ED and hypertension (60). In human subjects, hypertension has been correlated with a decrease in eNOS-mediated smooth muscle relaxation and it has been proposed that NO may be unable to overcome the sympathetic neural activity and other pro-vasoconstrictive mediators such as endothelins, neuropeptide Y, prostanoids, norepinephrine and angiotensin II that are involved in maintaining detumescence in the penis (64). Inasmuch as endothelial dysfunction has been associated with a variety of cardiovascular diseases including atherosclerosis, hypertension, and hypercholesterolemia, it was determined at the Second Princeton Consensus Conference in 2006 that ED is a telltale warning sign of silent vascular disease and that a man with ED without cardiac symptoms should be considered as an "at risk" cardiovascular patient until proven otherwise (65).

Prolonged and/or uncontrolled diabetes mellitus also has a strong association with ED (66). It has been reported that diabetic men are 3 times more

likely to develop ED compared to their non-diabetic counterpart (2, 66). In diabetic men, peripheral vasculopathy and neuropathy are strongly associated with the development of ED. Chronic hyperglycemia may lead to micro- and macro-vasculopathy, including endothelial dysfunction. Autonomic and peripheral neuropathies also develop commonly in individuals with diabetes mellitus (11). Diabetes-associated autonomic neuropathy can result in decreased parasympathetic neurotransmission to the corpora thereby decreasing the synthesis and release of vasodilators such as NO and ACh contained in the cavernosal nerves (67, 68).

Reduced NO bioavailability from oxidative stress is also prevalent in the pathophysiology of diabetes-associated complications. Both acute and chronic hyperglycemia can result in enhanced production of superoxide anion in the vascular endothelium (69-71). It has been reported that this mechanism can induce ED in animal models and that gene therapy with extracellular superoxide dismutase (EC-SOD) was able to restore erectile responses to cavernosal nerve stimulation in diabetic rats to control values (71). The risk factors for diabetes-associated ED include glycemic control, age, duration of the disease, and other common diabetic complications such as retinopathy. Hyperlipidemia, hypertension, and obesity are also all independent ED risk factors for type 2 diabetic men (11, 67).

Pelvic surgery has often been associated with the etiology of ED. Radical prostatectomy is one of the most commonly performed procedures for early stage prostate cancer (72). Virtually all patients that have undergone radical perineal or radical retropubic prostatectomy for pelvic malignancies report difficulty in achieving and maintaining an erection adequate for sexual relations following surgery (73). Tissue atrophy may also occur in many men post-prostatectomy with decreased penile length and circumference (74). A mechanism for this atrophic process has been proposed in the corporal tissue that is very similar to the Fas-mediated atrophy that occurs following spinal cord laceration and inhibition of neural stimulation to surrounding smooth muscle cells (75).

Johns Hopkins Urologist Patrick C. Walsh and his Dutch colleague Dr. Pieter Donker decreased the incidence of impotence following prostatectomy significantly with the development of a cavernosal nerve-sparing surgical technique, however the overall incidence of ED following this procedure still remains relatively high (55, 76). The primary reason this new technique has not eliminated post-operative ED has been attributed to the surgical margins, the degree of nerve sparing which these margins provide, and the unique manipulation of the nerve for individual surgical cases (55). Partial or complete nerve resection is required in some cases to completely remove the malignancy. Moreover, transient cavernosal nerve dysfunction, or "neuropraxia", is prevalent

in most patients following the nerve-sparing procedure for an average of 2 years (77).

V.   Current therapies for erectile dysfunction

Over the last three decades the biochemical basis of organic ED has had many significant breakthroughs and resulted in new, effective treatments and pharmacotherapies. The current standard of care for ED consists of lifestyle changes such as management of diet, diabetes, hypertension, and weight loss (11). When lifestyle changes do not improve outcomes with men and their sexual partners, pharmacotherapies may be used. These pharmacotherapies (particularly PDE-5 inhibitors) have become the mainstay for ED treatment and largely replaced vacuum devices and intrapenile prosthetics in men having problems attaining erection (78). There are two predominant delivery methods of erectogenic agents used to treat ED: intracavernosal injection and oral administration. Since the early 1980s intracavernosal injections of vasoactive agents has been the most reliable and effective therapy, however, this route of administration can cause pain, fibrosis of the penis at the site of repeated injections and systemic hypotension. Subsequently, intracavernosal injections have low patient compliance due to invasiveness and a higher incidence of priapism, or prolonged erections (79-81).

The most efficacious and commonly prescribed intracavernosal agent is a prostaglandin E_1 (PGE_1) analog synthesized from the precursor dihomo-α-linoleic acid known as alprostadil (79). Alprostadil binds G-protein coupled receptors on cavernosal smooth muscle cells, initiating an intracellular rise in cAMP (and decrease of intracellular plasma Ca^{2+}) through activation of adenylate cyclase (82, 83). The increasing concentration of cAMP levels result in increased kinase and myosin light chain phosphatase activity of the actin/smooth muscle/myosin cascade and subsequent smooth muscle relaxation. Alprostadil also attenuates adrenoreceptor vasoconstrictor tone by inhibiting release of norepinephrine through prejunctional receptors on adrenergic neurons (79). Alprostadil has a relatively short half-life of less than 10 minutes and is effective in providing an erection with sufficient rigidity for sexual intercourse in 73% of men suffering from ED, with a reduced risk of priapism when compared to intracavernosal injection of the nonselective cAMP/cGMP phosphodiesterase inhibitor papaverine (84, 85). The most common side effect associated with alprostadil is pain at the site of injection occurring in 30% of patients (86). Alprostadil also exists in a transurethral form which functions as a microsuppository that is inserted distally into the urethra after the patient urinates (86).

Oral pharmacotherapies have become the new mainstay of treatment for men suffering from ED, replacing intracavernosal injections and transurethral agents as first line therapies (11). The availability and ease of administration of

oral agents has also increased the frequency of men reporting sexual dysfunction to their physicians in recent years due to less fear associated with "swallowing a pill" and the increasing level of public awareness surrounding ED from marketing of these new therapies. As previously mentioned, PDE-5 is responsible for the breakdown of cGMP within cavernosal smooth muscle cells. PDE-5 inhibitors inhibit the breakdown of cGMP and subsequently enhance the erectile response. Interestingly, Dr. Rajfer and Ignarro's seminal study identifying NO as the as the endogenous molecule governing erection also reported that cavernosal smooth muscle relaxation responses could be enhanced by addition of the PDE-5 inhibitor zaprinast. These data provided the first evidence that PDE-5 inhibition could be used to enhance NO-mediated signaling in human penile tissue (29).

Sildenafil citrate (Viagra[®]) was the first oral PDE-5 inhibitor available for treatment of ED followed by vardenafil (Levitra[®]) and tadalafil (Cialis[®]). The action of these agents is based upon intact cavernosal nerves and corporal endothelium, because the initial physiologic release of NO is necessary to elicit the PDE-5 inhibitors' therapeutic effect. Therefore, the individual receiving treatment for ED must be sexually stimulated for the PDE-5 inhibitors to demonstrate efficacy. Sildenafil is 40% bioavailable after oral administration and is contraindicated in patients taking nitrates for angina pectoris due to the potential for a fatally severe drop in blood pressure (78, 87, 88). Flushing, headache, and visual disturbances (PDE-6 inhibition) have also been reported as

side effects with use of sildenafil citrate (79).

Vardenafil and tadalafil are newer PDE-5 inhibitors, and offer pharmacokinetic alternatives due to slight differences in their chemical ring structures (figure 7). Vardenafil, with a bioavailability of 15% after oral administration, may benefit from these structural changes through stronger binding interaction to the PDE-5 catalytic site (89). Tadalafil, however, differs in that the piperazine ring seen in the chemical structure of vardenafil and sildenafil is completely replaced with a hydantoin ring. Crossover trials have shown that men with ED prefer tadalafil to sildenafil by up to a 9 to 1 margin (90). This preference is increased in men with diabetes or hypertension, and is not significantly related to whether they hadpreviously taken sildenafil (90). Tadalafil has an increased half-life (17.5 hours) over sildenafil and vardenafil (4-5 hours), allowing patients a longer window of time for sexual activity after taking the medication (92). Because of this significantly prolonged half-life of tadalafil, a daily dosage may be taken to ensure a man with ED is always prepared for sexual activity.

Avanafil is a new, fast-acting PDE-5 inhibitor with less cross reactivity on other PDE isoforms and hence fewer side effects than other PDE-5 inhibitors utilized for treatment of ED (93, 94). Avanafil has the fastest therapeutic efficacy of clinically available PDE-5 inhibitors (95). A recently completed clinical study
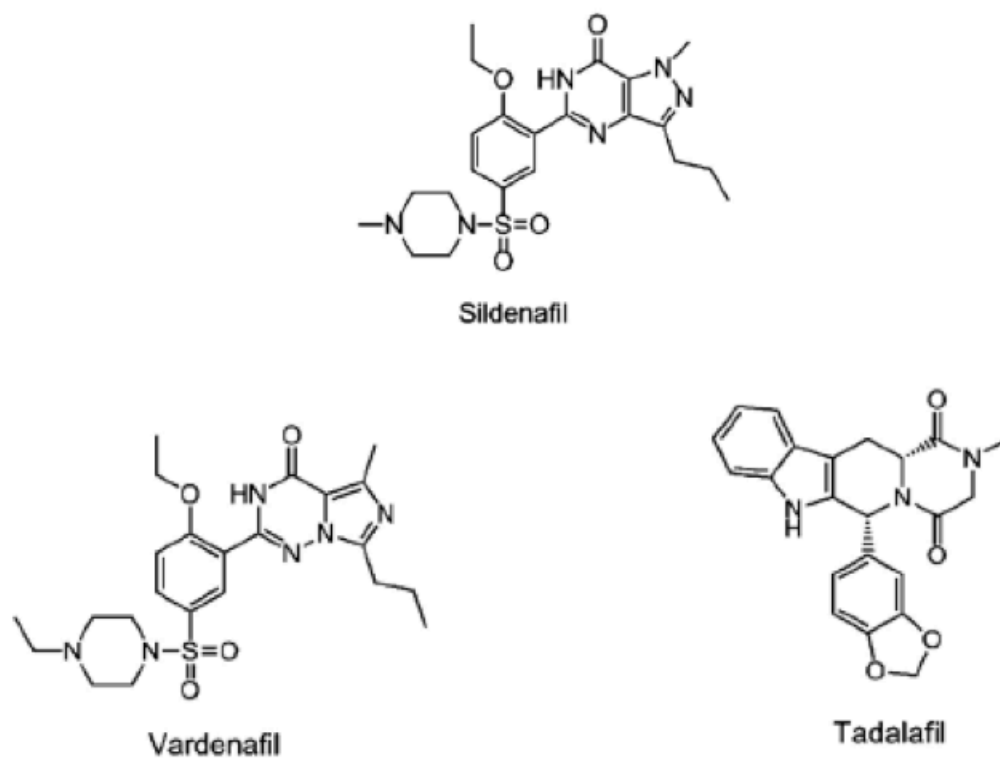
Figure 7. Chemical structures of the PDE-5 inhibitors sildenafil, vardenafil and tadalafil. Adapted from Patel *et al.*, J Pharm Biomed Anal 2013 (91).

involving 82 ED patients showed a peak erectile response observed 20-40

minutes following administration of avanafil, while increasing dosage from 100 to

200 mg enhanced efficacy of the PDE-5 inhibitor (96). Although these oral

therapies lead to higher compliance among individuals suffering from ED when

compared to invasive therapies such as intracavernosal injections of papaverine

or alprostadil, PDE-5 inhibitor therapy is not effective in disease states where NO

release may be significantly impaired.

VI.   Rho-kinase inhibitors

Penile vasodilation is responsible for mediating the erectile process and

inhibition of the vasoconstrictor Ca^{2+} sensitization pathway with inhibitors of Rho-

kinase offers a therapeutic alternative for the treatment of ED that does not

involve direct targeting of the NO/sGC/cGMP vasodilatory pathway (11). Western

blot analysis has verified the presence of RhoA/Rho-kinase in human penile

cavernosal smooth muscle (11, 97). Injection of the Rho-kinase inhibitor Y-27632

into the corpora cavernosa of rats pretreated with NOS inhibitors resulted in

erectile activity that was seemingly independent of endogenous NO release (11,

98). It has also been proposed that NO may act to inhibit the RhoA/Rho-kinase

pathway and that Rho-kinase may act to regulate NO-mediated vasodilation in

the normal erectile response (11, 99, 100). Increases in ICP in response to

intracavernosal injections of Y-27632 were observed without simultaneous

cavernosal nerve stimulation in animal experiments, providing evidence for a tonic role of the RhoA/Rho-kinase pathway in maintaining penile flaccidity (98). Rats administered intracavernosal injection with an adeno-associated viral gene dominant negative RhoA mutant (T19NRhoA) demonstrated enhanced erectile activity in response to cavernosal nerve stimulation providing further support for the role of RhoA in maintaining vasoconstriction in the penis (101). A mechanism for diabetes-associated ED was reported in which upregulated RhoA/Rho-kinase levels were found in the penile tissues of diabetic rats. Intracavernosal transfection with a dominant negative RhoA mutant into the diabetic rat penis restored erectile activity, cavernosal eNOS protein, NOS activity, and cGMP to levels observed in control animals (102). Hannan *et al.* have recently shown that RhoA/Rho-kinase signaling was upregulated in penile tissues following cavernosal nerve crush injury in the rat and that treatment with the prototypical Rho-kinase inhibitor, Y-27632, following nerve injury could significantly improve erectile function in these animals (103). Chronic administration of the Rho kinase inhibitor fasudil was shown to prevent vasculogenic ED and reduce levels of pelvic atherosclerosis in an animal model fed a high cholesterol diet (104). Another recent study has suggested that upregulated activity of RhoA/Rho-kinase in the diabetic rat penis enhances PTEN/Akt signaling and corporal apoptosis (11, 105). The study also provided evidence that chronic administration of the Rho-kinase inhibitor fasudil is more effective at reversing these pathologic changes in diabetic rats than insulin administration (105).

Inasmuch as the prototypical Rho-kinase inhibitors, such as Y-27632 or fasudil, have been reported to have potent erectile activity in the rat, these agents have also been reported to demonstrate inhibitory effects on a number of protein kinases, including protein kinase A, that can alter vascular smooth muscle function (106-108). Azaindole-1 is a highly selective Rho-kinase inhibitor with good pharmacokinetic properties that has little if any inhibitory effect on other cellular kinases (109). Although it has been reported that Rho-kinase inhibitors produce erectile activity that is independent of endogenous NO release and that ED may be associated with upregulation of the RhoA/Rho-kinase pathway, further research is needed to elucidate the relationship between Rho-kinase and NO in the acute erectile response.

VII.   Soluble guanylate cyclase stimulators and activators

Under normal conditions smooth muscle relaxation is initiated by interaction of NO with a heme iron on sGC's beta subunit, which breaks a histidine bond and activates the smooth muscle enzyme to generate cGMP from intracellular GTP. The need for therapies that bypass this NO-stimulating interaction but still target the sGC-cGMP pathway has led to the development and use of sGC stimulators and activators (110). A 1994 report by Ko *et al.* described a benzylindazole compound, YC-1, which prolonged the tail bleeding time in conscious mice and increased cGMP levels independently of NO (11,

111). Further studies have shown that YC-1 has vasodilator activity that synergizes with NO to potentiate vascular relaxation responses to NO donor agents (112). YC-1 was also shown to have erectile activity when injected into the corpora cavernosa of the rat (113). Research with this first "sGC stimulator" provided the framework for the next generation of stimulators with improved potency and selectivity for sGC, including the Bayer compounds BAY 41-2272 and BAY 41-8543 (114, 115).

Experiments with BAY 41-2272 and the detergent Tween-20, which can remove heme, showed that the sGC stimulator had no stimulatory effects on a heme-free sGC enzyme preparation in vitro (116). However, it was reported that BAY 41-2272 increased sGC activity 20-fold over baseline in the absence of NO and the response was potentiated further with the addition of the NO donor DEA/NO (11, 116). The mechanism of action for these stimulators has not been completely elucidated. It has been suggested that NO-YC-1 synergy exists because the sGC stimulator maintains the active conformation of sGC through stabilization of the nitrosyl-heme complex (11, 117-119). Furthermore, it was reported that the oxidized derivatives of both BAY 41-2272 and BAY 41-8543 maintain bioactivity in preclinical studies, suggesting a therapeutic role for the metabolites of these agents and their potential benefit in vivo (11, 120).

The properties of another sGC stimulator, A-350619, were investigated in a conscious rat model and induced penile erection shortly following intraperitoneal injection (121). BAY 41-2272 was administered to conscious rabbits IV and initiated a small erectile response similar to erectile response observed after IV administration of the PDE-5 inhibitor sildenafil, which was hypothesized to be weak due to lack of sexual stimulation and endogenous NO release (122, 123). The erectile responses to BAY 41-2272 in conscious rabbits were significantly enhanced when combined with administration of the NO donor sodium nitroprusside, demonstrating a synergy between the compound and exogenous NO (123). Similarly, our laboratory has shown that the sGC stimulator BAY 41-8543 has vasodilator activity in the pulmonary and systemic vascular beds of the intact chest rat that is significantly enhanced with small doses of sodium nitroprusside (124). A comparative study of sildenafil, an NO releasing PDE-5 inhibitor (NCX-911), and BAY 41-2272 in streptozotocin-induced diabetic rats reported that perineal muscle relaxation responses to NO were reduced and the diminished relaxation responses were potentiated by BAY 41-2272, but not by sildenafil or NCX-911 (11, 125).

In situations of severe oxidative stress, the iron on the heme motif of sGC can be oxidized or lost, decreasing the responsiveness of the enzyme to endogenously released NO, NO donor drugs or heme-dependent sGC stimulators (36, 116, 126). The finding that heme-free sGC could be activated by

pharmacological agents was first reported in 1982 by Ignarro and Wolin in their

studies with protoporphyrin IX (127). These results led to the concept that new

classes of agents could be effective at targeting sGC in pathological conditions

characterized by prolonged levels of severe oxidative stress when the enzyme is

unresponsive to NO. In these conditions the oxidized or heme-free enzyme can

be activated by sGC activators such as BAY 60-2770 (126). Stasch *et al.*

reported that sGC activators had greater ex-vivo vasodilator activity in diseased

human vessels when compared to healthy vessel preparations (128). A two-week

administration of the sGC activator BAY 60-2770 has also been reported to

ameliorate and normalize overactive bladder dysfunction in obese mice through

restoration of sGC-mediated micturition mechanisms (129).

In experimental animals sGC can be made insensitive to NO by treatment

with 1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one (ODQ), an agent that has

been demonstrated to oxidize the heme iron of sGC (108, 130-134). Zhao and

Marletta reported that ODQ had no effect on basal sGC activity but blocked NO-

mediated activation of the enzyme through oxidation of the ferrous heme on the

beta-subunit of sGC (134). These investigators also provided evidence that

activity of the enzyme could be restored by re-reducing the ODQ-treated sGC

with dithionite, providing further evidence that the pharmacologic activity of ODQ

is based upon oxidation of sGC's heme moiety (134). Our laboratory has recently

shown that the sGC activator BAY 60-2770 has significant vasodilator activity in

the pulmonary and systemic vascular beds of the intact chest rat that is enhanced when NOS is inhibited by L-NAME treatment or when sGC is inhibited by ODQ (132). These results, in addition to the findings of Stasch *et al.* showing that sGC activators have greater vasodilator activity on diseased vessel preparations when compared to healthy controls (128), suggest that sGC activators may be an effective treatment for ED associated with prolonged exposure to high levels of oxidative stress when other ED therapies have minimal therapeutic efficacy. Additionally, it has been shown that combination of the PDE-5 inhibitor vardenafil with the sGC stimulator BAY 60-4552 was able to partially restore erectile responses to cavernosal nerve stimulation (CNS) in a rat model of prostatectomy-associated nerve crush injury (135), however, the combination of an sGC activator with PDE-5 inhibitor therapy has not yet been reported in any studies on erectile function.

STATEMENT OF THE PROBLEM

Erectile dysfunction (ED) affects up to 50% of the male population from 40-70 years of age and is associated with ageing, diseases such as diabetes mellitus, and is also commonly observed in patients who have undergone pelvic surgeries for prostate cancer (2, 54, 55). Nitric oxide (NO) released from the cavernosal nerve and penile endothelium is the principal physiological mediator of cavernosal smooth muscle relaxation (17, 29-31, 136). NO binds to soluble guanylate cyclase (sGC) in smooth muscle and activates the enzyme to generate cyclic guanosine monophosphate (cGMP) from intracellular guanosine triphosphate (GTP). Increased levels of cGMP initiate cavernosal smooth muscle relaxation through activation of protein kinases that decrease intracellular calcium (Ca^{2+}) bioavailability, subsequently resulting in vasodilation and erection. Inasmuch as physiologic erection is reliant on sufficient synthesis and release of NO, the pathophysiology of ED is intimately related to decreased levels of bioavailable NO which may occur with iatrogenic nerve injury following prostatectomy and pathologic conditions of prolonged oxidative stress associated with many chronic diseases.

The cavernosal nerve is composed of adrenergic neurons that release norepinephrine, cholinergic neurons that release acetylcholine (ACh) and nonadrenergic, noncholinergic (NANC) neurons that release NO directly from the nerve terminal through activation of a neuronal nitric oxide synthase (nNOS) (10, 17, 36). Muscarinic receptors signal through activation of endothelial NOS (eNOS), and subsequent generation of NO, when ACh released from cholinergic nerves bind these receptors on the penile endothelium. Although it has been demonstrated that erectile responses are abolished after nonselective inhibition of NOS in animal studies (31), the contribution of the cholinergic component of the cavernosal nerve to the erectile response is not well understood and remains controversial. Reports on the effects of the muscarinic antagonist atropine during cavernosal nerve stimulation experiments vary from complete abolishment of the erectile response to no effect at all (43-50). Because the role of muscarinic receptors in the erectile response has not been definitively elucidated, further work is needed to determine the overall contribution of the cholinergic component of the cavernosal nerve to erection and to determine if new therapies may benefit patients with ED resulting from compromised cholinergic function.

Following prostatectomy and in situations of prolonged oxidative stress, the small amount of bioavailable NO is not sufficient to produce the levels of cGMP needed for smooth muscle relaxation and initiation of an erectile response. cGMP is hydrolyzed in penile tissues by cGMP phosphodiesterases, of which

type 5 phosphodiesterase (PDE-5) is the most abundant (137). The intracellular

cGMP concentration is determined by the rate of formation of the cyclic

nucleotide by sGC and the rate of metabolism of cGMP by phosphodiesterase

activity. The "gold standard" clinical treatment for ED is currently the PDE-5

inhibitor (11). It has been reported that more than half of ED patients benefit from

treatment with PDE-5 inhibitors (138). However, a number of patients do not

respond adequately to these oral agents (139). The causes of failure with the

PDE-5 inhibitors may include severity of ED, surgical procedures that cause

pelvic nerve damage, hypogonadism, incorrect drug dosage, and psychosocial

etiologies (139). PDE-5 inhibitor therapy requires a sufficient level of endogenous

NO formation in penile tissues for efficacy. However, in pathological conditions

where NO formation is severely impaired, PDE-5 inhibitors are not effective for

treatment of ED (11, 140).

Clinical trials with the PDE-5 inhibitor Viagra® reported that 50% of

patients after prostatectomy were not responsive to treatment (141). Similarly, it

was reported that 40% of diabetic patients did not respond to PDE-5 inhibitors for

treatment of ED (11, 141). Given the global prevalence of ED (5), it can be

estimated that the number of patients unresponsive to treatment with PDE-5

inhibitors to be in the millions. In addition to oral agents, other treatments for ED

include intraurethral therapy, intracavernosal injections, vacuum devices and

intrapenile prostheses (142-145). Intracavernosal injections have the highest

efficacy in patients who do not respond to PDE-5 inhibitor therapy; however, this therapy also has the lowest patient compliance because of the invasiveness of the injection procedure and the high incidence of priapism (144). Improved therapies that are effective in pathophysiologic situations of decreased NO bioavailability are needed for these patient populations.

Rho-kinase inhibitors, sGC stimulators and sGC activators offer three new interventions that may demonstrate efficacy in treating ED resulting from pathophysiologic conditions of low NO formation or bioavailability. Rho-kinase inhibitors exert their vascular effects through cessation of a Ca^{2+} sensitization mechanism that enhances smooth muscle contraction caused by the small GTPase RhoA and its downstream effector, Rho-kinase. These agents decrease myosin light chain kinase activity and increase activity of smooth muscle phosphatases involved in smooth muscle contraction (146). The role of Rho-kinase inhibitors and their relationship to endogenous NO in the acute erectile response has not been adequately elucidated. Although it has been reported that the RhoA/Rho-kinase pathway is upregulated over time in several pathological models of ED (102-105) and that erectile responses to Rho-kinase inhibitors are not dependent on endogenous NO (98), many of the initial studies with Rho-kinase inhibitors were performed with agents that have "off-target" effects on other cellular kinases that have been shown to alter vascular tone, such as cAMP-dependent protein kinase (PKA) (107, 146). Azaindole-1 is a new Rho-

kinase inhibitor that is highly selective for Rho-kinase and has little if any effect

on other cellular kinases (109). Utilization of azaindole-1 with various inhibitors of

NO-mediated signaling may be able to offer insight into the relationship between

Rho-kinase and endogenous NO in the acute erectile response.

sGC stimulators are NO-independent, heme-dependent agents that

directly activate sGC and increasing the catalytic activity of the enzyme (59). sGC

stimulators can also potentiate vasodilator responses to small amounts of NO

(147). The observation that sGC stimulators produce synergistic responses with

low levels of NO indicates that these therapies may be effective for treatment of

ED in situations of nerve injury where NO bioavailability is reduced and PDE-5

inhibitors do not produce an adequate erectile response. Although sGC

stimulators can act independently of NO as well as synergize with bioavailable

NO to enhance the generation of cGMP, these agents require a reduced heme

moiety (Fe^{2+}) on sGC to activate the enzyme and thus may not be effective in

conditions of enhanced oxidative stress (36, 148).

Severe oxidative stress can oxidize the ferrous iron (Fe^{2+}) in the heme

binding motif of sGC to a ferric state (Fe^{3+}), which decreases the sensitivity of the

enzyme to NO or heme-dependent sGC stimulators (128). In extreme

pathological conditions when NO formation or bioavailability is impaired and sGC

is oxidized, rendering an incapability of interaction with NO or sGC stimulating

agents, sGC activators represent a novel form of therapy that may restore normal erectile function. NO-independent, heme-independent sGC activators have vasodilator activity under control conditions, however, it has been shown that the pharmacologic effect of these agents is greatly enhanced when sGC is oxidized or heme-free (126). It is therefore possible that sGC activators can promote adequate erectile function in patients with severe ED that remain unresponsive to more traditional therapies.

It is the purpose of these studies to determine the cholinergic contribution of the erectile response to cavernosal nerve stimulation in the anesthetized rat. The role of Rho-kinase in the acute erectile response will also be determined. Moreover, using specific inhibitors to block components of NO-mediated signaling and a nerve crush injury model of ED, these studies were performed to determine if erectile responses could be rescued with sGC-targeted pharmacotherapies in conditions of low NO bioavailability.

HYPOTHESES

1.   Muscarinic receptor activation plays a significant role in the erectile

        response to cavernosal nerve stimulation in the anesthetized rat.

2.   RhoA/Rho-kinase modifies NO-mediated erectile responses and when

        Rho-kinase is pharmacologically inhibited, potentiated erectile responses

        to endogenous NO will occur.

3.   Pharmacological targeting of sGC can induce erectile responses under

        control conditions and improve erectile responses when NO formation or

        bioavailability is decreased or when sGC is oxidized.

4.   sGC activators may be used in combination with PDE-5 inhibitor therapy

        to significantly enhance erectile activity in conditions of oxidative stress.

SPECIFIC AIMS

The proposed research will examine the hypothesis that muscarinic receptor activation plays a significant role in the erectile response of the rat and pharmacological agents that directly target sGC or Rho-kinase may be used to enhance erectile activity when cholinergic neurotransmission or other NO signaling pathways are impaired. The specific aims of the study were:

1. **To determine the role of muscarinic receptor activation in mediating the erectile response to cavernosal nerve stimulation.** These studies will investigate the effect of the cholinergic antagonist atropine sulfate on erectile responses to cavernosal nerve stimulation. Additionally, the same dose of atropine will be used to assess erectile and systemic vascular responses to intracavernosal and intravenous injection of both acetylcholine and sodium nitroprusside.

2. **To determine the effect of Rho-kinase inhibitors and stimulators and activators of sGC on erectile function in physiologic and pathophysiologic conditions.** These experiments will investigate the

erectile response to Rho-kinase inhibitors and sGC stimulators and activators under control conditions. Additionally, the effect of these agents will be investigated when muscarinic receptors are blocked with atropine, when all NOS isoforms are inhibited with L-NAME, when sGC is oxidized with ODQ or when pelvic nerves are damaged by nerve crush injury.

3. **To determine the effect of combination therapy for ED using an sGC activator and a new PDE-5 inhibitor.** These studies will investigate the effect of administration of a new, fast acting PDE-5 inhibitor (avanafil) on erectile responses to the sGC stimulator BAY 60-2770 under control conditions and when NO-mediated vasorelaxation is attenuated with the sGC inhibitor, ODQ.

MATERIALS AND METHODS

I. <u>General</u>

    A. <u>Experimental Animals</u>

Sprague-Dawley rats (Charles River) were used for all of the studies. Animals were housed in the Tulane University School of Medicine Vivarium in a temperature controlled room monitored daily by the veterinary staff, with a 12 h light-dark cycle and given free access to standard chow and water. The Institutional Animal Care and Use Committee of Tulane University School of Medicine approved the experimental protocol used in all of these studies, and all procedures were conducted in accordance with institutional guidelines.

    B. <u>Measurements of hemodynamic values in the rat</u>

There are many biochemical, histological and physiologic similarities between the corpora cavernosa of the rat and the cavernosal tissues of the human (149). Both species demonstrate adrenergic, cholinergic and nonadrenergic, noncholinergic innervation of the penis and share similar second

messenger pathways of vasodilation in the penis, which makes the rat a good model to study mechanisms of penile erection and new treatments for ED. Furthermore, because of the ease of accessibility of the cavernosal nerve following laparotomy, the rat serves as a good translational model to evaluate new therapies for iatrogenic ED. To investigate treatments for prostatectomy-associated ED, cavernosal nerve injury models have been developed in the rodent (108, 150).

For these experiments, adult male Sprague-Dawley rats were anesthetized with Inactin (thiobutabarbital), 100 mg/kg intraperitoneally. Supplemental doses of Inactin were given as needed to maintain a uniform level of anesthesia. Body temperature was maintained with a heating lamp. The trachea was cannulated with a short segment of PE-240 tubing to maintain a patent airway, and the left carotid artery was catheterized with PE-50 tubing for measurement of systemic arterial pressure. ICP was measured with a 25-gauge needle inserted into the left crus of the penis and connected to PE-50 tubing filled with heparinized saline. Systemic arterial pressure and ICP were measured with Namic Perceptor DT pressure transducers and a data acquisition system (Biopac MP 100A-CE, Santa Barbara, CA). ICP, systemic arterial pressure, and mean systemic arterial pressure (MAP) obtained by electronic averaging were continuously recorded and were displayed and stored on a Dell PC (figure 8). The left jugular vein was catheterized with PE-50 tubing for the systemic
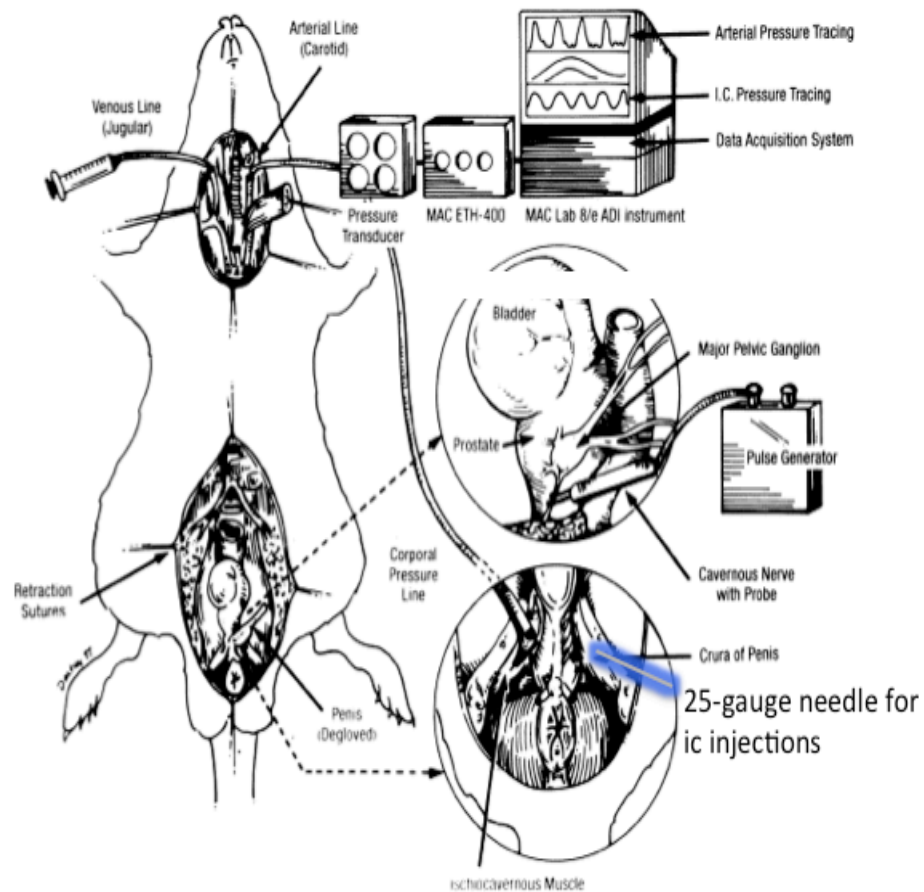
Figure 8. Experimental model in the rat for measurement of agonist-induced and cavernosal nerve stimulation-induced erectile responses. Adapted from Rehman *et al.*, Urology 1998 (151).

administration of drugs and fluids. A 25-gauge needle connected to PE-50 tubing was placed in the right crus of the penis for administration of drugs. Maximal ICP in response to intracavernosal injection of the vasodilator agents or in response to cavernosal nerve stimulation was measured at the peak of the ICP pressure increase. The maximum ICP divided by the MAP at that point in time was used to normalize for variations in systemic driving pressure into the penis. The area under the ICP curve (AUC) and duration of the increase in ICP were measured to characterize the total erectile response.

Cardiac output was measured by the thermodilution technique. A 1.5F thermistor microprobe (Columbus Instruments, Columbus, OH) was positioned in the aortic arch by introduction through the left carotid artery to measure blood temperature. A known volume of indicator 0.9% NaCl (0.2 mL) at room temperature was injected into the left jugular vein catheter with its tip near the right atrium. The Cardiomax III (Columbus Instruments) cardiac output computer measured the changes in blood temperature and determined the cardiac output, which was stored on the PC. Methemoglobin levels were measured in 85 µl arterial blood samples removed from the carotid artery catheter and were analyzed with a Radiometer NPT7 series analyzer (Copenhagen).

In experiments in which pharmacological agents, diluted to a consistent volume of 200 µl were utilized, all intracavernosal injection of drugs were made

when ICP was at baseline value. The erectile effects of a single injection were monitored until ICP returned to pre-injection value. For experiments with multiple injections, the next injection was made after a 5 minute period from the end of the preceding erectile response to ensure a stable ICP baseline throughout the entirety of the experiment. Injection of 200 µl of all vehicles used in experiments had no significant effect on ICP.

C. <u>Cavernosal nerve stimulation experiments</u>

For nerve stimulation experiments, the bladder and prostate were exposed through a midline abdominal incision. The cavernosal nerve was identified posterolateral to the prostate on one side, and a stainless steel bipolar stimulating electrode was placed on the nerve. The cavernosal nerve was stimulated with square wave pulses at frequencies of 2, 4, 8, 10, and 16 Hz, at 5 V and a pulse width of 5 milliseconds for 60 seconds per intervention with a Grass Instruments SD9 Stimulator (Quincy, MA). A rest period of at least 3 min was allowed between nerve stimulation trials and similar results were obtained with 5- and 10-min rest periods. Erectile responses to cavernosal nerve stimulation were quantified in a similar manner to intracavernosal injection of the vasodilator agents, with measurement of the peak ICP, maximum ICP/MAP and area under the erectile curve (AUC: mmHg x seconds) (Fig. 9).
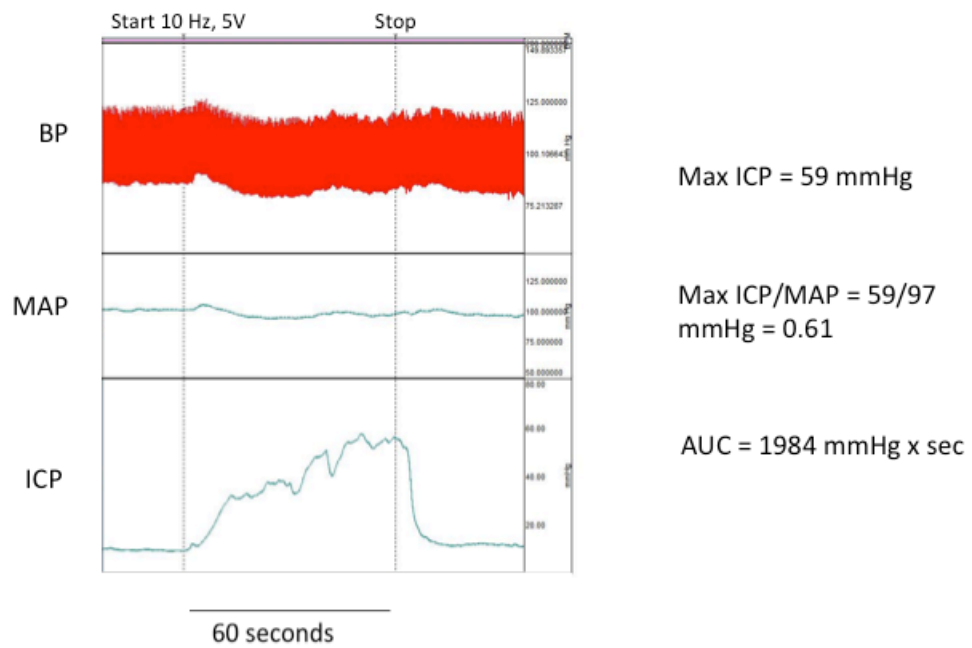
Figure 9. Record of an erectile response to cavernosal nerve stimulation at 10 Hz in the anesthetized rat.

D. <u>Cavernosal nerve crush injury experiments</u>

Acute nerve crush injury experiments were performed using three 15 second applications of a 3-inch surgical forcep to the cavernosal nerve 5 mm distal from the major pelvic ganglion. For chronic crush experiments, rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), the prostate was exposed through a midline laparotomy, and the cavernosal nerves were identified. Bilateral nerve crush injury was performed by applying three 15 second applications of a 3-inch surgical forcep to the cavernosal nerves 5 mm distal from the major pelvic ganglion. A two-layered closure was performed on the animals using a 4–0 Vicryl suture. Thirty days after bilateral cavernosal nerve injury, erectile function was assessed as described above.

II. <u>Experimental Design</u>

A. <u>Analysis of the effect of muscarinic receptor antagonism on erectile responses</u>

The innervation of corpora cavernosa includes an adrenergic component, a cholinergic component and a nonadrenergic, noncholinergic (NANC) component. The contribution of cholinergic innervation and muscarinic receptor activation to the erectile response has not been clearly defined (43-50). In these

experiments, the erectile response to electrical stimulation of the cavernosal

nerve at a range of frequencies (2-16 Hz) before and after intravenous (IV)

administration of the muscarinic receptor antagonist atropine (1 mg/kg) was

assessed through measurement of changes in ICP, max ICP/MAP to normalize

for variation in systemic driving pressure, and area under the erectile curve

(AUC). To determine the efficacy and selectivity of muscarinic receptor

antagonism and the effects of atropine administration on downstream effectors of

NO-mediated signaling, control experiments were performed with intracavernosal

and IV injections of acetylcholine (ACh) and the NO donor sodium nitroprusside,

respectively.

B. <u>Analysis of the effect of NOS inhibitors on erectile responses to cavernosal nerve stimulation in the anesthetized rat</u>

Experiments with the nonselective NOS inhibitor N-nitro-L-arginine methyl

ester (L-NAME) and the selective nNOS inhibitor 7-nitroindazole (7-NI) were

performed to determine the effect of these agents on responses to cavernosal

nerve stimulation. The nerve was stimulated at a range of frequencies (2-16 Hz)

to establish frequency-dependent erectile responses under control conditions.

After completing the range of stimulations, either 7-NI (10 mg/kg) or L-NAME (50

mg/kg) was administered IV and the range of stimulation frequencies was

repeated and compared to pretreatment values of change in ICP, max ICP/MAP

and AUC.

C. <u>Analysis of the effect of ODQ on NO-mediated and NO-independent erectile responses</u>

In the first set of experiments erectile responses to intracavernosal injection of the NO donors sodium nitroprusside (1 µg/kg) and DEA/NO (10 µg/kg) were assessed before and after intracavernosal administration of ODQ (2 mg/kg). Changes in ICP, the ratio of ICP/MAP at maximum ICP, and AUC were measured to characterize the effect of the sGC inhibitor on exogenous NO-mediated erectile responses.

In the second set of experiments, the cavernosal nerve was stimulated at a range of frequencies (2-16 Hz) before and 15 minutes after intracavernosal administration of ODQ (2 mg/kg). Changes in ICP, the ratio of ICP/MAP at maximum ICP, and area under the ICP curve were measured to characterize the effect of the sGC inhibitor on endogenous NO-mediated erectile responses. A small volume of arterial blood was obtained before and after ODQ administration to measure off-target oxidizing effects of ODQ on other heme-containing proteins such as hemoglobin.

In the third set of experiments, the effect of intracavernosal administration

of 2 mg/kg ODQ was evaluated on three non-NO based vasodilator agents. In these experiments intracavernosal injection of the Rho-kinase inhibitor fasudil (30 µg/kg), the nonselective beta adrenergic agonist isoproterenol (100 ng/kg) and the tyrosine-kinase inhibitor imatinib (10 mg/kg) were given before and after intracavernosal administration of ODQ to determine if inhibition of sGC altered the erectile response to any of these agents.

In the final set of experiments, the effect of ODQ administration on erectile responses to the NO-independent, heme-dependent stimulator BAY 41-8543 were assessed. Changes in ICP, maximum ICP/MAP and AUC were measured in response to intracavernosal injection of 10 µg/kg of the sGC stimulator before and after intracavernosal administration of 2 mg/kg ODQ.

D. <u>Analysis of the erectile response to Rho-kinase inhibitors</u>

In the first set of experiments, responses to intracavernosal injections of azaindole-1 (1-100 µg/kg), fasudil (1-100 µg/kg), and sodium nitroprusside (0.1-10 µg/kg) were compared, and changes in ICP, the ratio of ICP/MAP at maximum ICP, the duration of the increase in ICP, and AUC were measured.

In the second set of experiments, the effects of the nNOS inhibitor 7-NI and the muscarinic receptor antagonist atropine on the response to cavernosal

nerve stimulation at 10 Hz and the Rho-kinase inhibitors were investigated. The

cavernosal nerve was stimulated before and 15 minutes after the IV

administration of 7 NI (10 mg/kg); then, atropine (1 mg/kg IV) was administered

and the nerve was re-stimulated to evaluate the combined effect of muscarinic

receptor antagonism and nNOS inhibition on the response to cavernosal nerve

stimulation at 10 Hz. Responses to intracavernosal injections of azaindole-1 and

fasudil (30 µg/kg) were investigated after treatment with 7-NI and atropine.

The last set of experiments investigated the effects of azaindole-1 and the

prototypical Rho-kinase inhibitor, Y-27632, on the erectile response to cavernosal

nerve stimulation after acute nerve crush injury. The nerve was crushed with

three 15-second applications of a 3-inch forceps and then stimulated through the

site of injury to induce attenuated endogenous erectile responses. Next, the

nerve was stimulated with a simultaneous 30 µg/kg intracavernosal injection of

either azaindole-1 or Y-27632 to determine the acute relationship between

endogenous NO and Rho-kinase inhibitors in pathological conditions of nerve

crush injury.

E. <u>Analysis of erectile responses to the sGC stimulator BAY 41-8543 under
control and pathophysiological conditions</u>

sGC stimulators are NO-independent agents that have been reported to

demonstrate marked vasodilator activity in vivo in the systemic and pulmonary vascular beds (124, 126, 132, 148). The erectile responses to the first sGC stimulator characterized, YC-1, were reported by Mizusawa *et al.* in 2002 (113). In the first set of experiments erectile responses to intracavernosal injections of BAY 41-8543 (0.1–100 µg/kg) and sodium nitroprusside (0.01–10 µg/kg) were compared and changes in ICP, MAP, the ratio of ICP/MAP at maximum ICP, the duration of the increase in ICP and area under the ICP curve (AUC) were measured when doses of the agents were expressed on a nmol/kg basis to take differences in molecular weight into account.

In the second set of experiments, the cavernosal nerve was stimulated before and 10 minutes after administration of atropine, 1 mg/kg IV, and then an intracavernosal injection of BAY 41-8543, 0.1 µg/kg, was administered in the atropine-treated animal to determine if a low dose of the sGC stimulator could restore the erectile response to cavernosal nerve stimulation after muscarinic receptor blockade to control value.

In the third set of experiments, the interaction between exogenous NO and BAY 41-8543 was investigated. In these experiments intracavernosal injection of a low dose of the NO donor sodium nitroprusside (0.01 µg/kg) was given alone and together with BAY 41-8543 (0.3 µg/kg) and responses to intracavernosal injections of sodium nitroprusside and BAY 41-8543 when given alone and in

combination were compared.

In the fourth set of experiments, the interaction between endogenously released NO and BAY 41-8543 was investigated. In these experiments the effect of intracavernosal injection of BAY 41-8543 (0.1 µg/kg) during stimulation of the cavernosal nerve at 2 Hz was investigated.

In the last set of experiments the effects of BAY 41-8543 on the response to cavernosal nerve stimulation was investigated after acute nerve crush injury to determine if BAY 41-8543 had a beneficial effect in this pathologic model of ED. The nerve was crushed with three 15-second applications of a 3-inch forceps and then stimulated through the site of injury to induce attenuated erectile responses to electrical stimulation of the cavernosal nerve. Intracavernosal injection of 1 µg/kg BAY 41-8543 was administered concomitantly with stimulation of the crushed nerve to determine if a low dose of the sGC stimulator could rescue responses to cavernosal nerve stimulation under pathologic conditions of nerve injury.

F. <u>Analysis of erectile responses to the sGC activator BAY 60-2770 under control and pathophysiological conditions</u>

In pathophysiologic conditions associated with chronic levels of increased

oxidative stress, the heme moiety of sGC may become oxidized and non-responsive to NO or sGC-stimulating agents (36). sGC activators target the NO-insensitive sGC to increase the catalytic activity of the oxidized enzyme in smooth muscle (26). In the first set of experiments, responses to intracavernosal injections of the NO-independent, heme-independent sGC activator BAY 60-2770 (1-300 ng/kg) were evaluated. Changes in ICP, MAP, maximum ICP/MAP and AUC were measured to characterize the erectile activity of the sGC activator.

In the next set of experiments, erectile responses to intracavernosal injection of the sGC activator BAY 60-2770 (100 ng/kg) were evaluated before and after treatment with the nonselective NOS inhibitor L-NAME (50 mg/kg IV). Pilot studies demonstrate that this dose of L-NAME decreases the erectile response to cavernosal nerve stimulation by approximately 90%. Erectile responses to intracavernosal injection of BAY 60-2770 were assessed by changes in ICP and total area under the erectile curve.

In the third set of experiments, intracavernosal injections of the sGC activator BAY 60-2770 (1-10 ng/kg) were performed before and after the intracavernosal administration of ODQ (2 mg/kg). Intracavernosal doses of the NO donor sodium nitroprusside were also given before and after ODQ to confirm inhibition of NO-mediated vasorelaxation responses prior to injection of the sGC activator

In the last set of experiments the effects of BAY 60-2770 on the response to cavernosal nerve stimulation was investigated after acute and chronic nerve crush injury to determine if BAY 60-2770 had beneficial effects in a post-prostatectomy model of ED. Intracavernosal injections of 100 ng/kg BAY 60-2770 were administered to determine if erectile responses to the sGC activator were altered in pathologic conditions of nerve injury.

G. <u>Analysis of the erectile response to combination therapy with an sGC activator and a PDE-5 inhibitor</u>

PDE-5 inhibitors enhance and prolong smooth muscle relaxation by decreasing the activity of the enzyme that metabolizes cGMP. However, these agents are also dependent on adequate levels of cGMP to initiate erection. With prolonged levels of increased oxidative stress, sGC may become non-responsive to endogenous NO and PDE-5 inhibitors may not be sufficient to treat ED. Avanafil is a new, faster acting PDE-5 inhibitor with less cross reactivity to other PDE isoforms and may have fewer side effects than other PDE-5 inhibitors on the market for treatment of ED (93).

Combination therapy with the PDE-5 inhibitor vardenafil and the sGC stimulator BAY 60-4552 was reported to partially restore erectile responses to cavernosal nerve stimulation in a rat model of nerve injury (135), however,

combination therapy with an sGC activator and a PDE-5 inhibitor has not yet

been reported in any studies on erectile function. To determine the effect of

combination therapy with a PDE-5 inhibitor and an sGC activator, intracavernosal

injection of the NO donor sodium nitroprusside (positive control) and BAY 60-

2770 (10 ng/kg) were performed before and after IV administration of avanafil (1

mg/kg). To determine the effect of combination therapy in a model of chronic

oxidative stress where sGC is oxidized and unresponsive to endogenous NO, a

similar experiment was performed with ODQ and avanafil administration between

intracavernosal injections of BAY 60-2770.

III. Drug Preparation

BAY 60-2770 (4-({(4-Carboxybutyl)[2-(5-fluoro-2-{[4'-(trifluoromethyl)-

biphenyl-4-yl]methoxy}phenyl)ethyl]amino}methyl)benzoic acid) and BAY 41-

8543 (2-[1-(2-fluorophenylmethyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-5(4-

morpholinyl)-4,6-pyrimidinediamine) were dissolved in Transcutol-Cremophor EL-

0.9% NaCl solution (10:10:80). ODQ (1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-

one) (Cayman) was also dissolved in the Transcutol-Cremophor vehicle. The

dose of ODQ used in these experiments was determined from pilot experiments.

Inactin (thiobutabarbital sodium salt), sodium nitroprusside, DEA/NO

(Diethylammonium (Z)-1-(N,N- diethylamino)-diazen-1-ium-1,2-diolate), L-NAME

(N-nitro-L-arginine methyl ester), atropine sulfate and isoproterenol hydrochloride

(Sigma-Aldrich) were dissolved in 0.9% NaCl. Imatinib mesylate (Novartis) and avanafil (Vivus) were dissolved in deionized water titrated to a pH of 5. Azaindole-1 (6-chloro-N4-{3,5-difluoro-4-[(3-methyl-1H-pyrrolo[2,3-b]pyridin-4-yl)oxy]-phenyl}pyrimidine-2,4-diamine) was dissolved in Transcutol-Cremophor EL-0.9% NaCl solution (10:10:80). Fasudil (LC Laboratories, HA-1077) and Y-27632 dihydrochloride (Tocris Bioscience, (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide dihydrochloride) were dissolved in 0.9% NaCl solution. 7-NI (7-nitroindazole) was dissolved in a 1.0 % dimethyl sulfoxide solution (Sigma-Aldrich). All drugs were kept on ice in light-resistant glass bottles and working solutions were prepared on a frequent basis. The vehicles for all drugs used in these studies had no significant effect on vascular pressures.

IV. Statistical Analysis

All data are expressed as mean value ± standard error of the mean (SEM) and were analyzed using a one-way analysis of variance (ANOVA), an unpaired Student's t-test or a paired Student's t-test. A p value < 0.05 was used as the criterion for statistical significance.

RESULTS

I.   <u>Effect of muscarinic receptor antagonism on the response to cavernosal</u>
      <u>nerve stimulation</u>

The role of muscarinic receptor activation in mediating the response to
cavernosal nerve stimulation was investigated and stimulation of the nerves
innervating the corpora at 2-16 Hz produced stimulus-frequency related
increases in ICP, with the peak increase occurring at 10 Hz (Fig. 10).
Intracavernosal pressure increased 17 ± 5, 49 ± 8, 53 ± 9, 62 ± 6, and 59 ± 6
mmHg at stimulus frequencies of 2, 4, 8,10, and 16 Hz, respectively (Fig. 10).
The duration of each nerve stimulation trial was 1 minute, with a rest period of at
least 3 minutes between subsequent stimulations and similar data was obtained
with 5- or 10-minute rest periods. The increase in ICP/MAP and AUC in response
to cavernosal nerve stimulation at stimulation frequencies of 4, 8, 10, and 16 Hz
was significantly attenuated after administration of atropine in a dose of 1 mg/kg
IV (Fig. 10).
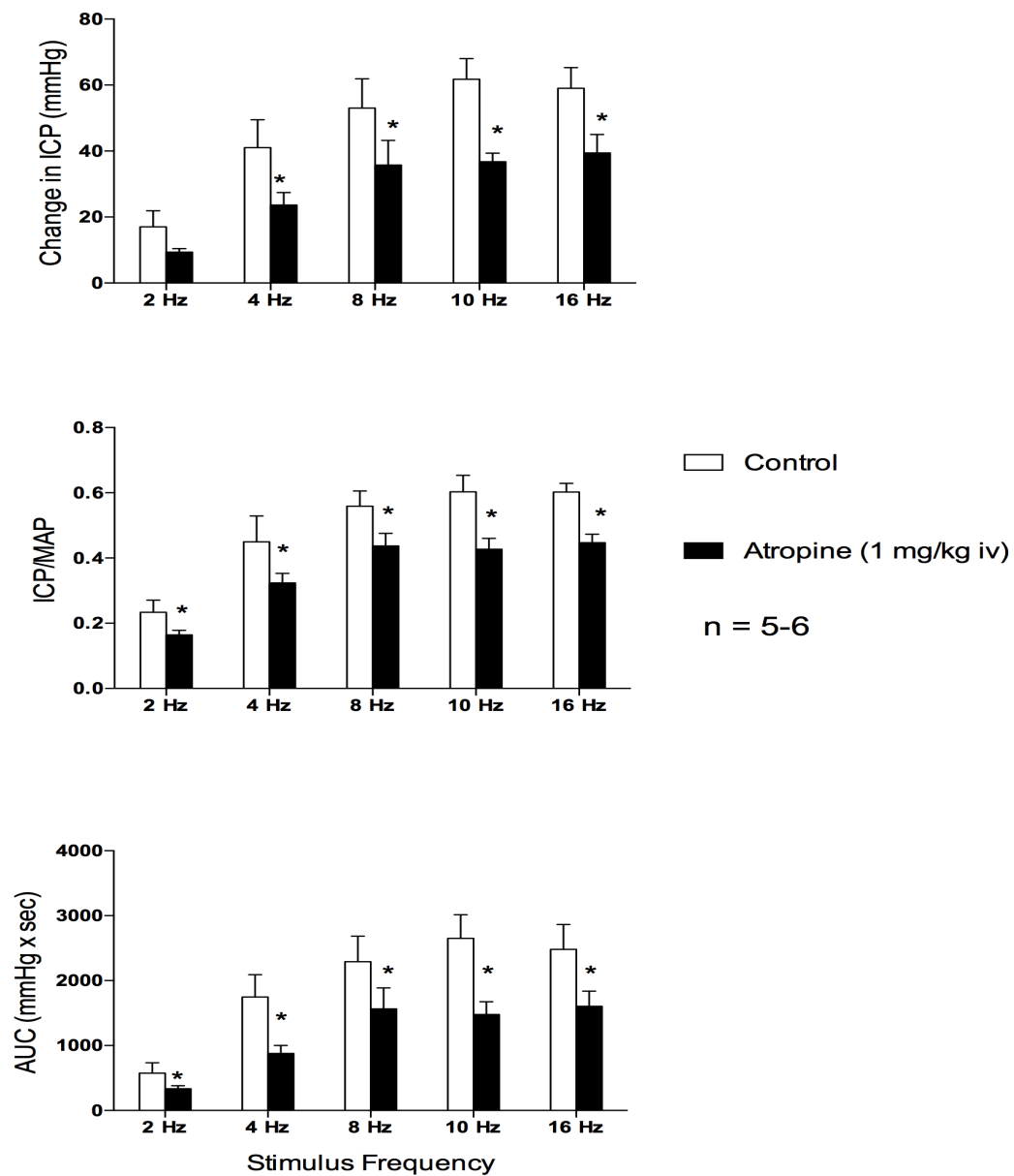
Figure 10. Bar graphs showing the effect of atropine 1 mg/kg IV on erectile responses to cavernosal nerve stimulation in the anesthetized rat. n indicates number of experiments, * indicates p < 0.05 when compared to corresponding control. Adapted from Lasker *et al.*, J Sex Med 2013 (152).

The increase in ICP in response to intracavernosal injection of ACh was reduced significantly after treatment with atropine, 1 mg/kg IV, whereas the erectile response to intracavernosal injection of sodium nitroprusside was not changed after administration of the muscarinic antagonist (Fig. 11A, B). Similarly, the decrease in MAP in response to IV injection of ACh was significantly attenuated after the administration of atropine 1 mg/kg IV while the decrease in MAP in response to IV injection of sodium nitroprusside was not altered (Fig. 11C, D). The systemic vasodilator responses to IV injections of ACh occurred without significant changes in cardiac output or heart rate (figure 12). These data indicate that the increase in ICP and decrease in MAP in response to injection of ACh are mediated by the activation of muscarinic receptors in the corpora cavernosa and in the systemic vascular bed. The absence of an effect of atropine on erectile or systemic vasodepressor responses to sodium nitroprusside while significantly attenuating erectile and systemic vasodilator responses to injection of ACh indicate that the muscarinic receptor blockade is effective and specific in the penile and systemic vascular beds.
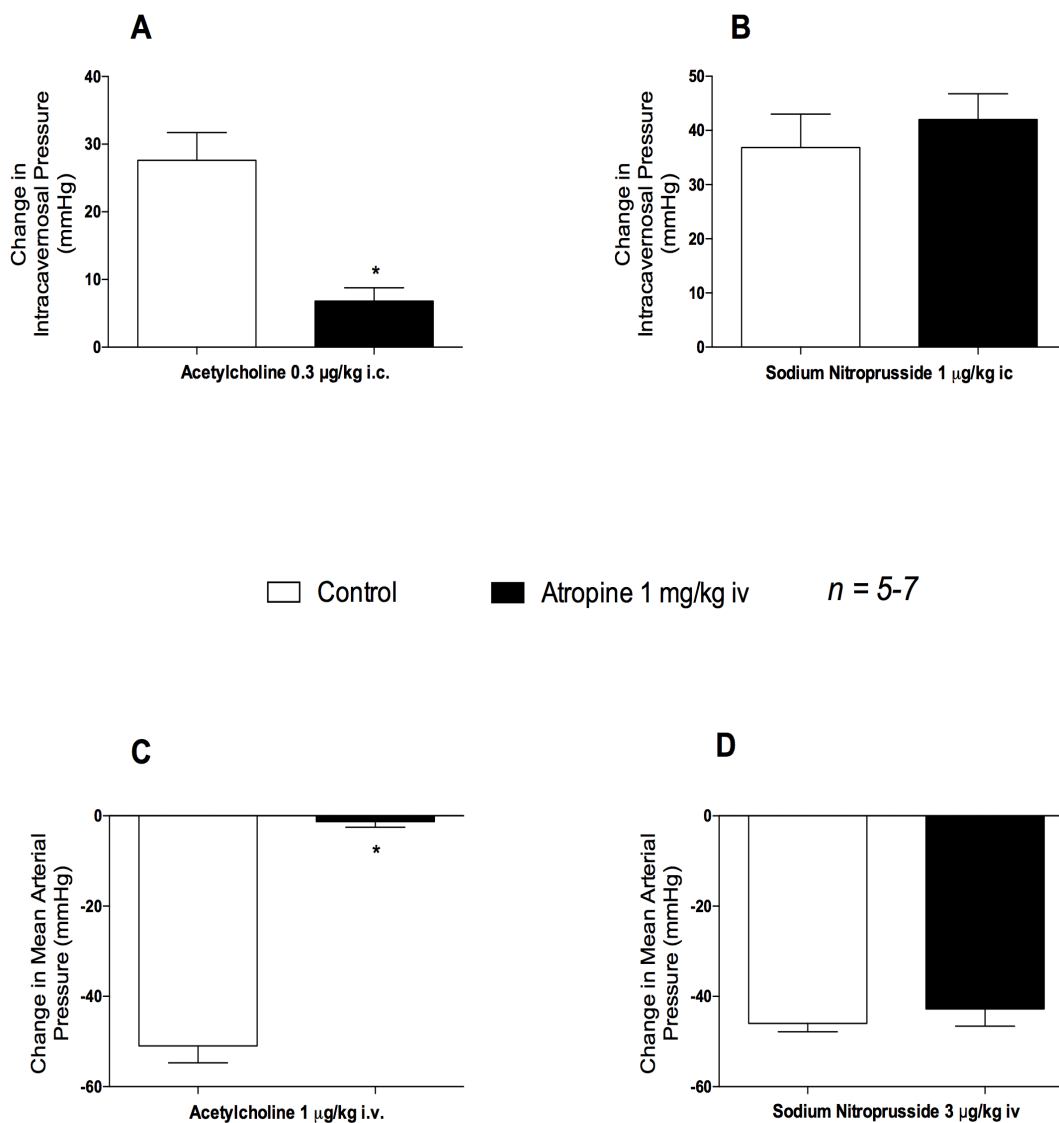
Figure 11. Bar graphs comparing erectile and systemic vasodilator responses to intracavernosal and intravenous injections of acetylcholine before and after administration of atropine (A & C, respectively). Bar graphs comparing erectile and systemic vasodilator responses to intracavernosal and intravenous injections of sodium nitroprusside before and after administration of atropine (B & D, respectively). n indicates number of experiments, p < 0.05 when compared with corresponding control. Adapted from Lasker *et al.*, J Sex Med 2013 (152).

Figure 12. Bar graphs showing the effects of intravenous injections of acetylcholine on systemic arterial pressure (A), cardiac output (B), and heart rate (C) before (open bars) and after (black bars) treatment with atropine. n indicates number of experiments, * indicates $p < 0.05$ when compared to control. Adapted from Lasker *et al.*, J Sex Med 2013 (152).

In order to provide information on the mechanism by which ACh induces erectile responses, the effect of the nonselective NOS inhibitor L-NAME on the response to intracavernosal injection of ACh was investigated. Following administration of L-NAME in a dose of 50 mg/kg IV the erectile response to intracavernosal injection of ACh was significantly attenuated (Fig. 13). The data with atropine and L-NAME suggest that the response to intracavernosal injection of ACh is mediated by the activation of muscarinic receptors and the release of NO through a NOS-mediated mechanism in cavernosal endothelium.

The role of nNOS activation in mediating the response to cavernosal nerve stimulation was investigated and stimulation of the nerves innervating the corpora at 2, 4, 8, 10, and 16 Hz produced stimulus-frequency related increases in ICP, with the peak increase occurring at 10 Hz (Fig. 14). The duration of the nerve stimulation trial was 1 minute, at which time the increase in ICP reached a steady value with a rest period of at least 3 minutes between subsequent stimulations and similar data was obtained with 5- or 10-minute rest periods. The increases in ICP, ICP/MAP and AUC in response to cavernosal nerve stimulation at stimulation frequencies of 2, 4, 8, 10, and 16 Hz was significantly attenuated at all stimulation frequencies after administration of the nNOS inhibitor 7-nitroindazole in a dose of 10 mg/kg IV (Fig. 14).
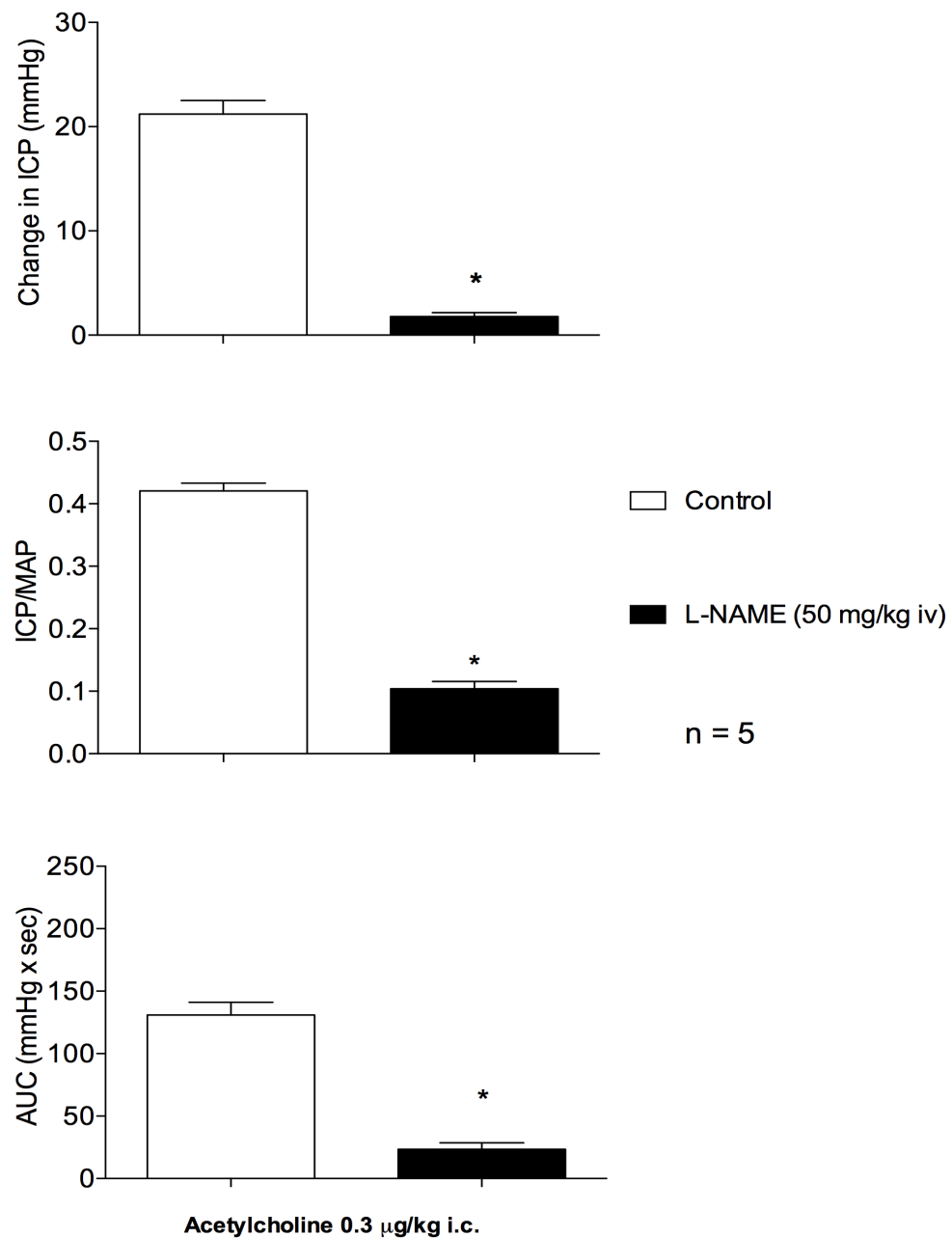
Figure 13. Bar graphs comparing erectile responses to acetylcholine before and after L-NAME treatment. n indicates number of experiments, * indicates p < 0.05 when compared to controls. Adapted from Lasker *et al.*, J Sex Med 2013 (152).
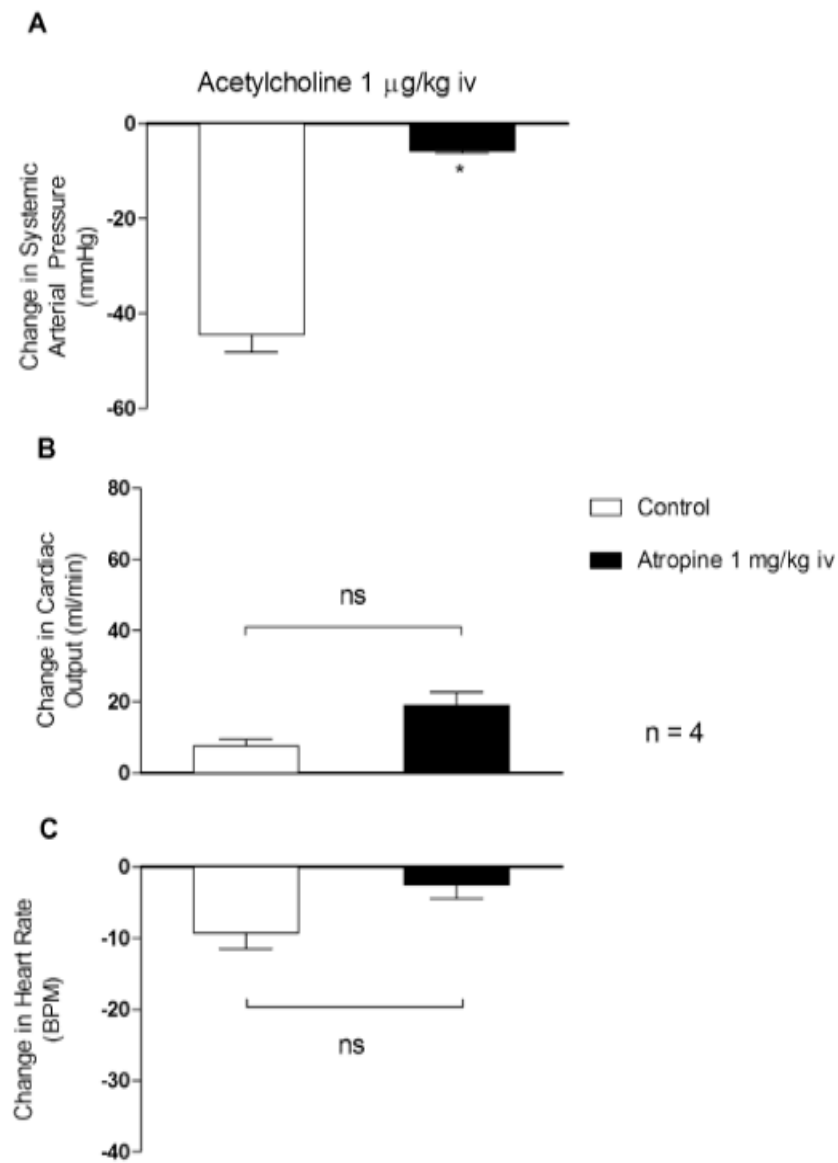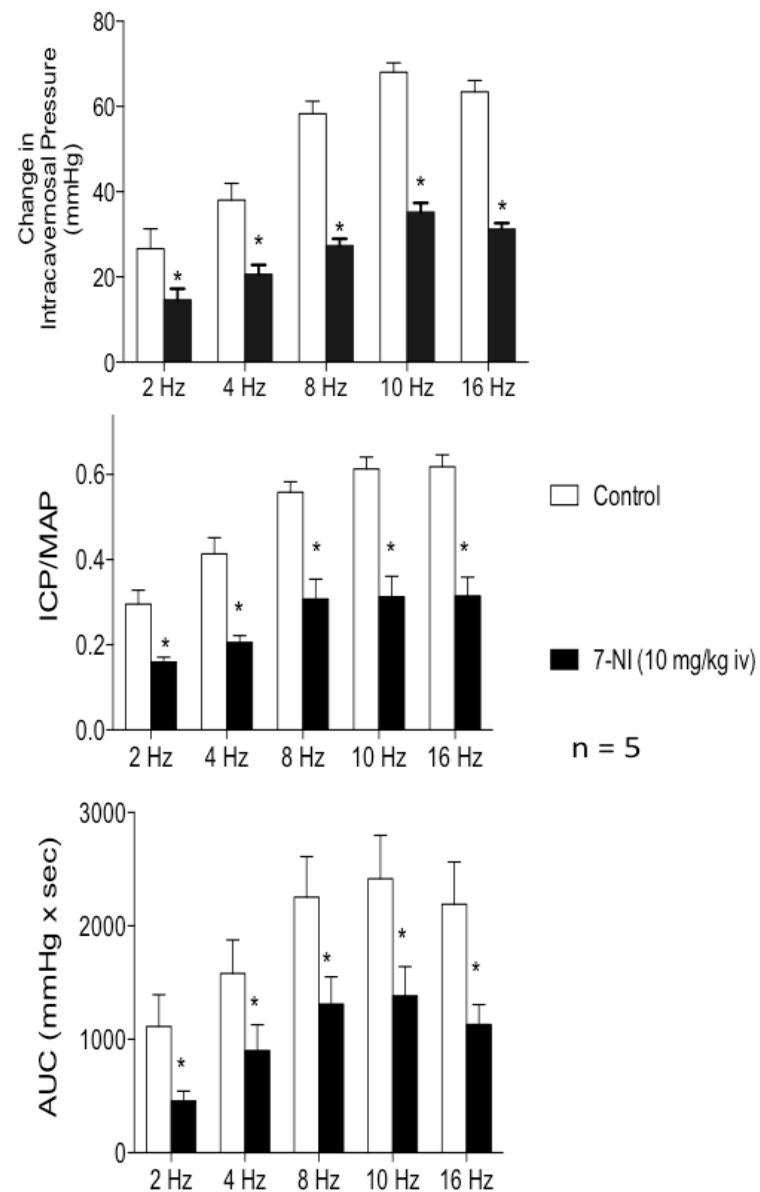
Figure 14. Bar graphs showing the effect of 7-nitroindazole on erectile responses to cavernosal nerve stimulation in the anesthetized rat. n indicates number of experiments, * indicates $p < 0.05$ when compared to corresponding control. Adapted from Lasker *et al.*, Urology 2013 (108).

To investigate the effect of inhibition of NOS on the erectile response to cavernosal nerve stimulation, experiments were performed with the nonselective NOS inhibitor L-NAME. Treatment with L-NAME, 50 mg/kg IV, significantly attenuated the increases in ICP, ICP/MAP, and AUC in response to cavernosal nerve stimulation at all stimulus frequencies tested (Fig. 15). The duration of the nerve stimulation trial was 1 minute, at which time the increase in ICP reached a steady value with a rest period of at least 3 minutes between subsequent stimulations and similar data was obtained with 5- or 10-minute rest periods. The increase in ICP/MAP and AUC in response to cavernosal nerve stimulation at stimulation frequencies of 2, 4, 8, 10, and 16 Hz was decreased significantly at all stimulation frequencies after administration of the nonselective NOS inhibitor (Fig. 15). It must be mentioned that in these experiments the administration of L-NAME, 50 mg/kg IV, caused an increase in systemic driving pressure (MAP) of approximately 30-40 mmHg in each animal. Therefore, in order to quantify the total NOS contribution to the erectile response in terms of ICP/MAP and AUC, experiments with a systemic infusion of a vasodilator that could normalize the driving pressure (MAP) to control value would need to be performed.
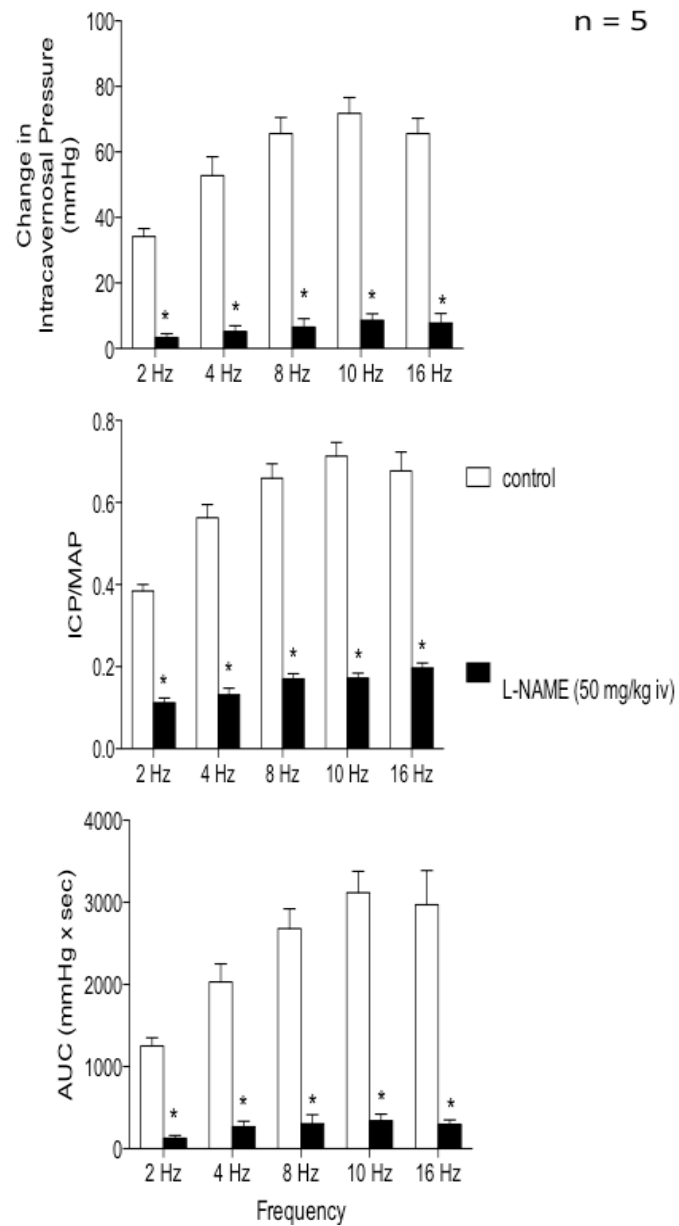
Figure 15. Bar graphs illustrating the effect of L-NAME treatment on erectile responses to cavernosal nerve stimulation. n indicates number of experiments, * indicates $p < 0.05$ when compared to corresponding control. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).

II.   <u>Effect of the soluble guanylate cyclase inhibitor ODQ on erectile responses in the rat</u>

ODQ is an agent that has been shown to inhibit the activation of sGC by NO through oxidation of the heme iron on the beta subunit of the enzyme (134). In the present study, the intracavernosal administration of ODQ in a dose of 2 mg/kg attenuated the increases in ICP, max ICP/MAP and AUC in response to intracavernosal injection of the NO donors sodium nitroprusside and DEA/NO as shown in figure 16. The effect of ODQ treatment on the erectile response to cavernosal nerve stimulation was investigated and these data are summarized in figure 17. Intracavernosal injection with ODQ, 2 mg/kg, significantly reduced the increases in ICP, ICP/MAP, and AUC at all stimulus frequencies studied. These data indicate that the responsiveness of sGC in the corpora cavernosa to endogenous NO (neurogenically released) and exogenous NO (released from pharmacological agents) is attenuated by ODQ treatment in the anesthetized rat.
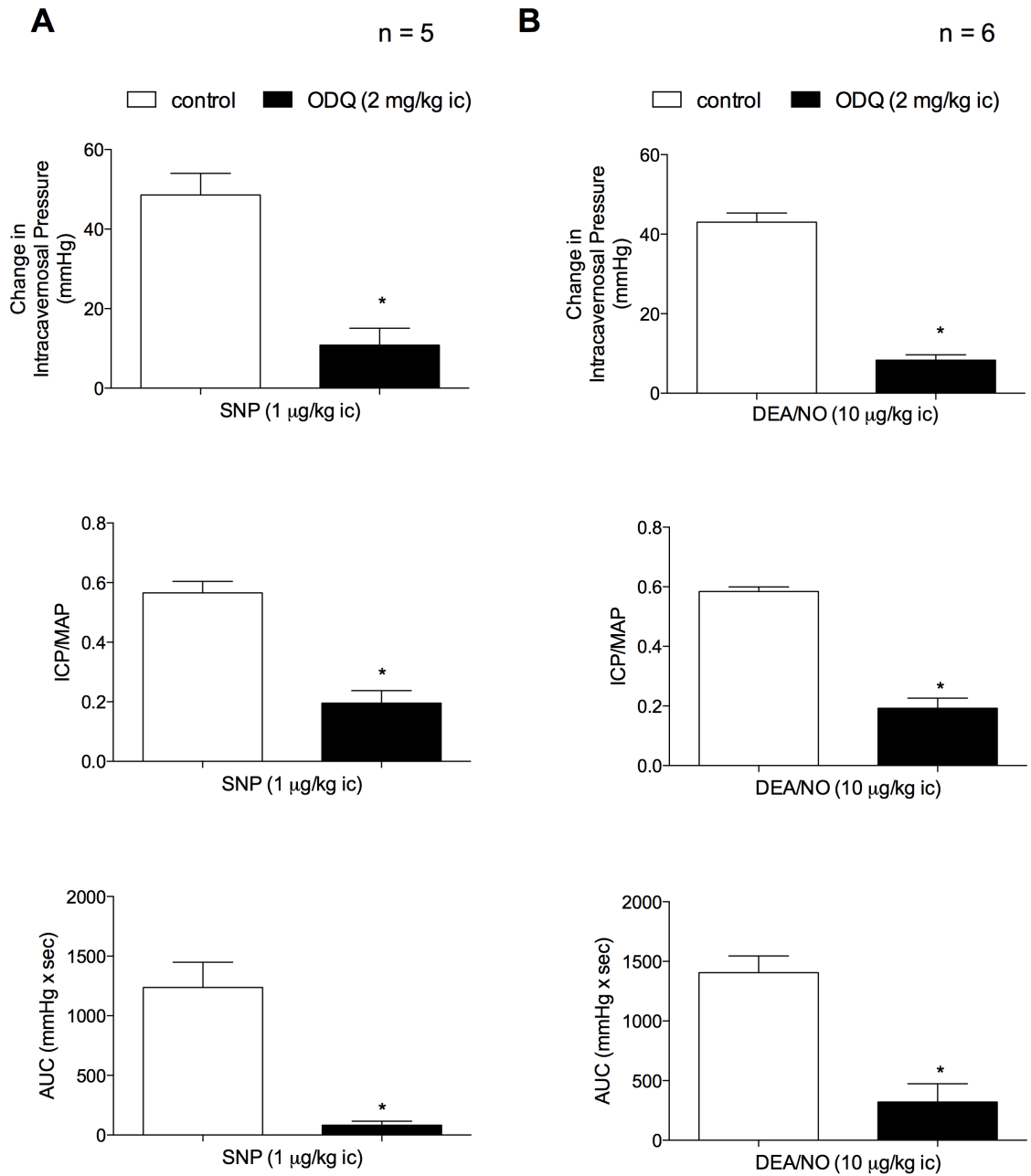
Figure 16. Bar graphs showing the effect of treatment with ODQ on erectile responses to intracavernosal injection of the NO donors sodium nitroprusside (A) and DEA/NO (B). n indicates number of experiments, * indicates p < 0.05 using student's paired t-test. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).
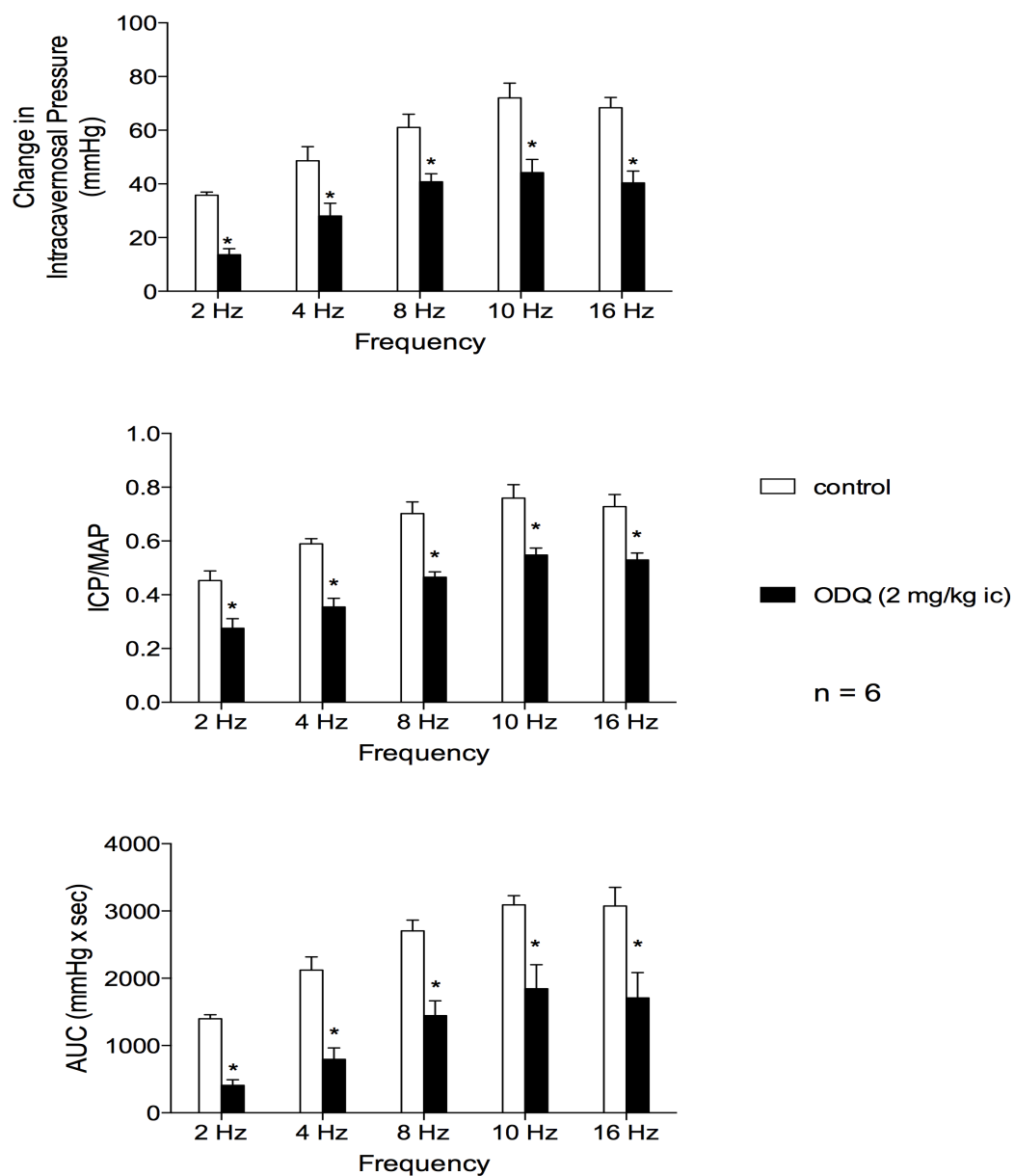
Figure 17. Bar graphs illustrating the effect of ODQ treatment on the erectile response to cavernosal nerve stimulation in the anesthetized rat. n indicates number of experiments, * indicates $p < 0.05$ using student's paired t-test. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).

The inhibitory effect of ODQ on NO-mediated erectile responses was evaluated in experiments with isoproterenol and imatinib. The intracavernosal injection of 100 ng/kg isoproterenol or 10 mg/kg imatinib produced significant increases in ICP, ICP/MAP, and AUC, that were not significantly changed after treatment with ODQ (Fig. 18). These data indicate that the inhibitory effect of ODQ on NO-mediated erectile responses is specific or not off-target since the erectile response to agents that increase ICP by diverse mechanisms was examined and these results indicate that ODQ did not alter erectile responses mediated by activation of beta adrenergic receptors (isoproterenol) or inhibition of tyrosine kinase signaling (imatinib).

Because ODQ can oxidize other heme proteins including hemoglobin, the effect of ODQ treatment on methemoglobin levels was investigated and the results show that intracavernosal administration of ODQ, in a dose that inhibited erectile responses to NO donors and cavernosal nerve stimulation (2 mg/kg), had no significant effect on methemoglobin values in arterial blood (control metHb = $1.77 \pm 0.42\%$ vs. ODQ-treated metHb = $2.32 \pm 0.47\%$, $p < 0.05$, $n = 6$).
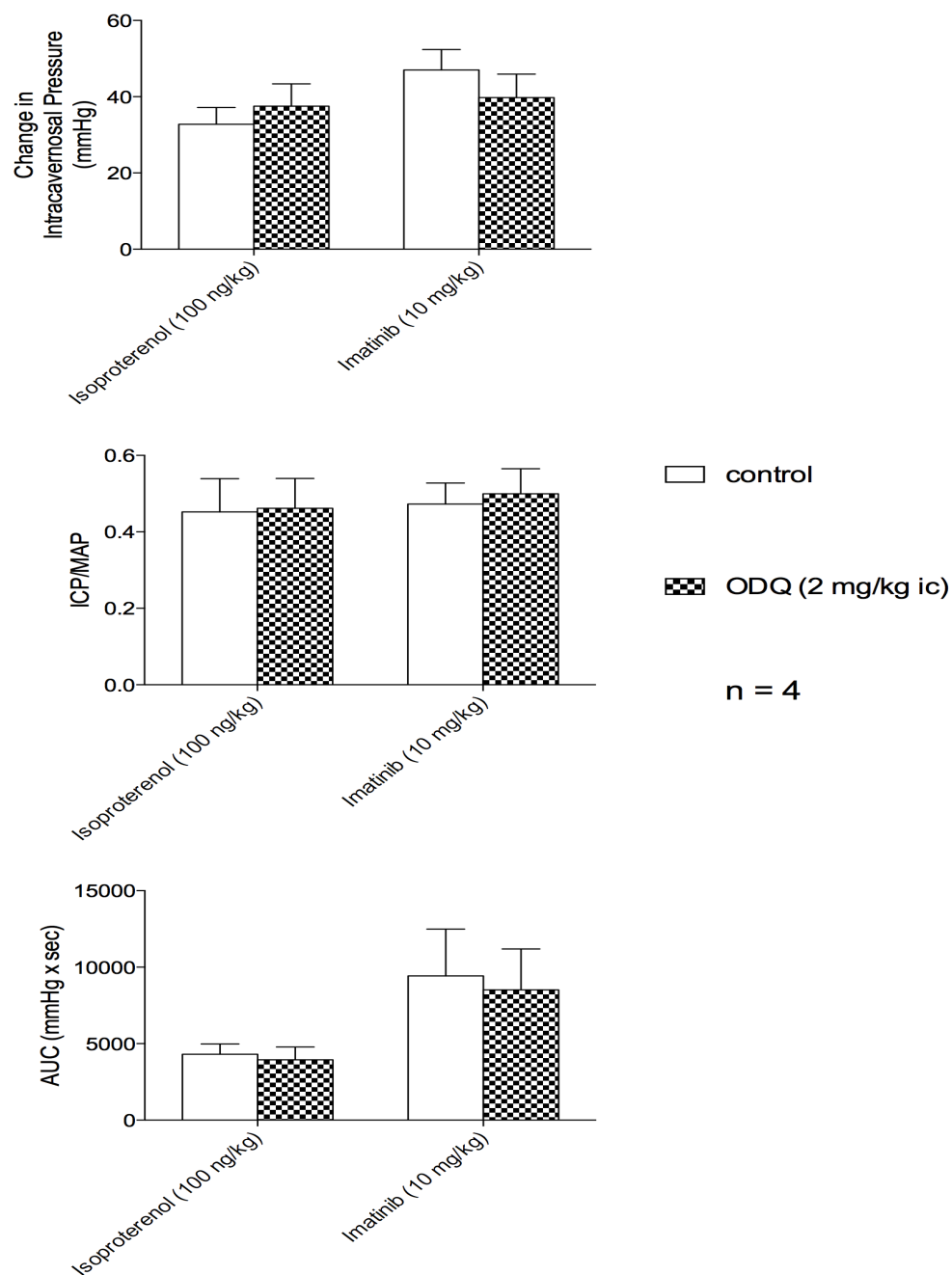
Figure 18. Bar graphs comparing the effect of ODQ treatment on the erectile responses to intracavernosal injections of the beta-adrenergic agonist isoproterenol and the tyrosine kinase inhibitor imatinib. n indicates number of experiments, * indicates $p < 0.05$ using student's paired t-test. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).

III.   <u>Role of Rho-kinase in NO-mediated erectile responses</u>

Erectile responses to the Rho-kinase inhibitor azaindole-1 were investigated in the anesthetized rat and intracavernosal injections of azaindole-1 and a prototypical Rho-kinase inhibitor, fasudil, in doses of 1 to 100 µg/kg, produced dose-related erectile activity (Fig. 19). Doses are expressed in nmol/kg to account for differences in molecular weight between the drugs. Responses to intracavernosal injection of azaindole-1 were rapid in onset (20-40 seconds) and long in duration (6598 seconds at the highest dose studied). Erectile responses to azaindole-1, the prototypical Rho-kinase inhibitor fasudil, and the NO donor sodium nitroprusside were compared (Fig. 19A-D). These experiments show that the increases in ICP/MAP in response to intracavernosal injections of azaindole-1 and fasudil were similar, however, the AUC and duration of the erectile response in response to injections of azaindole-1 were significantly greater when compared with fasudil (Fig. 19 C&D). Both Rho-kinase inhibitors produced similar decreases in MAP when injected into the corpora.
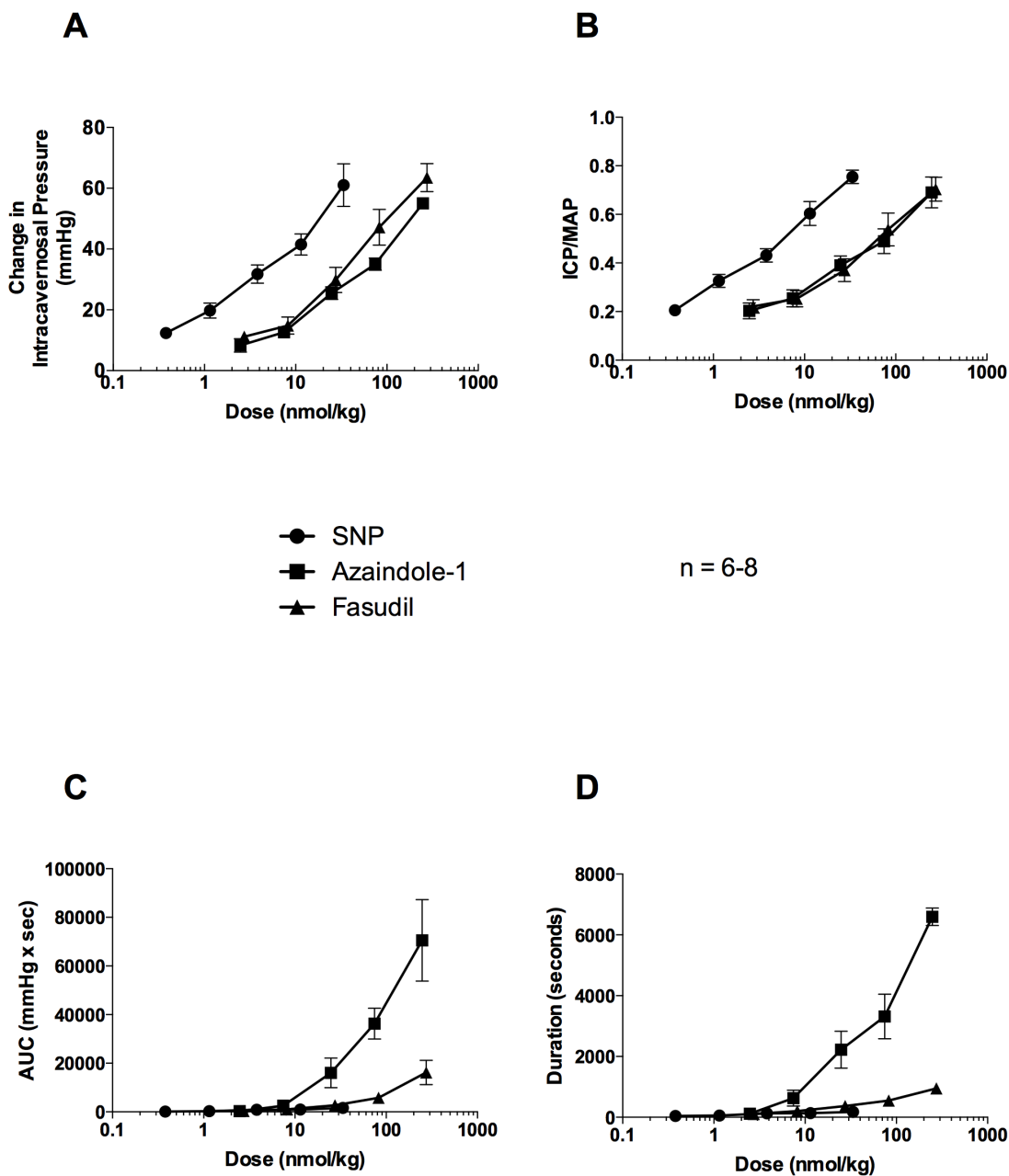
Figure 19. Line graphs comparing erectile responses to intracavernosal injections of a wide range of doses of azaindole-1, fasudil, and sodium nitroprusside (SNP). n indicates number of experiments. The error bars indicate the standard error of the mean value. Adapted from Lasker *et al.*, Urology 2013 (108).

Erectile responses to azaindole-1 and fasudil were investigated in experiments where nNOS activity was inhibited by 7-NI and cholinergic neurotransmission was antagonized with atropine. IV injections of 7-NI (10 mg/kg) and atropine (1 mg/kg) reduced the response to cavernosal nerve stimulation at 10 Hz by approximately 80% (Fig. 20A). After the response to cavernosal nerve stimulation was significantly attenuated by nNOS inhibition and muscarinic receptor antagonism, the intracavernosal injection of azaindole-1 or fasudil at a dose of 30 µg/kg produced erectile responses that were not significantly different than responses to the 2 Rho-kinase inhibitors recorded under control conditions (Fig. 20B).

The effect of the sGC inhibitor ODQ on erectile responses to the NO donor sodium nitroprusside (positive control), azaindole-1, and fasudil was investigated in the anesthetized rat. The intracavernosal injection of sodium nitroprusside (1 µg/kg) produced an erectile response that was significantly attenuated after treatment with ODQ (2 mg/kg) (Fig. 21A). After administration of ODQ, the increases in ICP, ICP/MAP, and AUC in response to intracavernosal injections of azaindole-1 or fasudil (30 µg/kg) were not different than responses obtained under control conditions (Fig. 21B), indicating that both Rho-kinase inhibitors' erectile activity is not affected when sGC, and subsequent cGMP signaling, is inhibited.
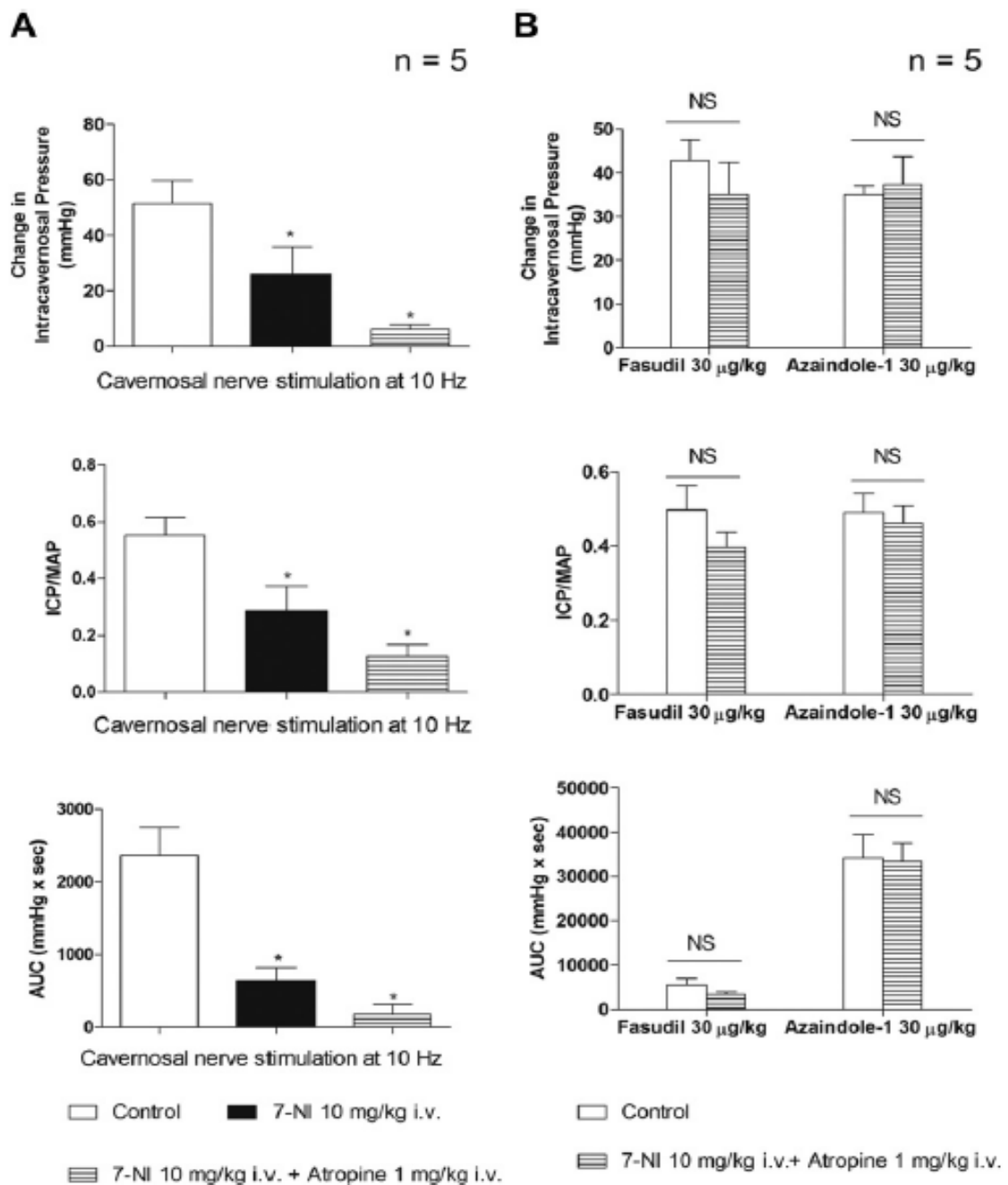
Figure 20. (A) Bar graphs comparing erectile responses to cavernosal nerve stimulation at 10 Hz before and after administration of 7-nitroindazole (7-NI) and the combination of 7-NI + atropine (B) Bar graphs compare erectile responses to intracavernosal injection of azaindole-1 and fasudil before and after treatment with 7-NI and atropine. n indicates number of experiments, * indicates $p < 0.05$ and NS indicates no significant difference. Adapted from Lasker *et al.*, Urology 2013 (108).
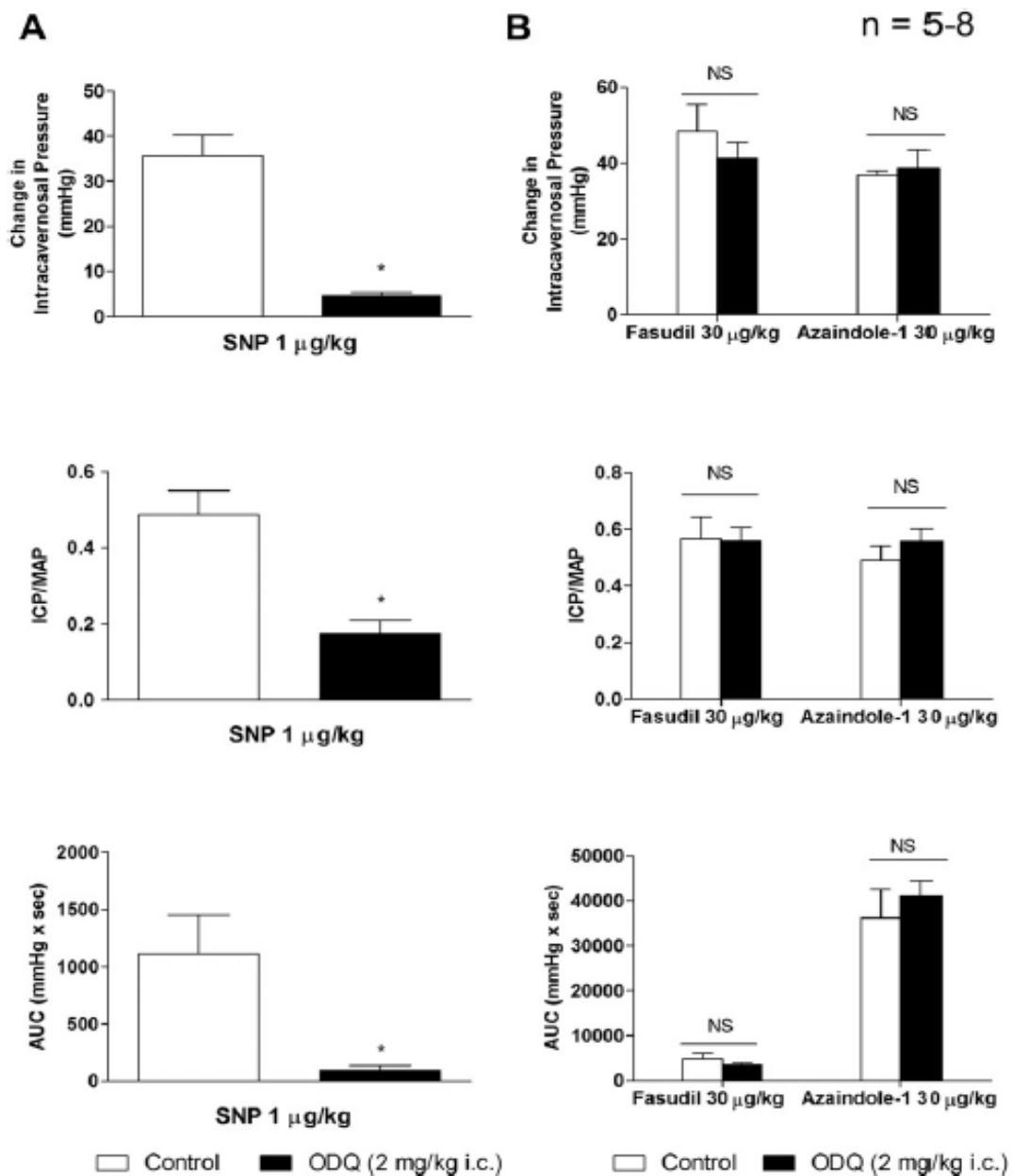
Figure 21. (A) Bar graphs comparing erectile responses to intracavernosal injection of sodium nitroprusside before and after treatment with ODQ. (B) Bar graphs comparing erectile responses to intracavernosal injection of azaindole-1 and fasudil before and after treatment with ODQ (2 mg/kg). n indicates number of experiments, * indicates $p < 0.05$ and NS indicates no significant difference. Adapted from Lasker *et al*., Urology 2013 (108).

The effect of azaindole-1 and another prototypical Rho-kinase inhibitor, Y-27632, on erectile function after acute cavernosal nerve injury was investigated, and these data are summarized in figure 22. Acute nerve injury induced by forcep compression of the cavernosal nerve decreased the erectile response to electrical stimulation of the cavernosal nerve by 89%, and responses did not spontaneously return to control value during the 2-hour period of the experiments (Fig. 22A). The intracavernosal injection of azaindole-1 (30 µg/kg) or Y-27632 (30 µg/kg) in animals after nerve injury produced increases in ICP that were not significantly different from the responses observed in control animals. Moreover, the increase in ICP in response to the intracavernosal injection of azaindole-1 (30 µg/kg) or Y-27632 (30 µg/kg) during cavernosal nerve stimulation at 10 Hz after nerve injury produced increases in erectile parameters that were not significantly different from the algebraic sum of the erectile responses to each intervention when applied separately (Fig. 22 B,C). These data indicate that the increases in ICP to endogenous NO and Rho-kinase inhibitors are additive and that the Rho-kinase inhibitors do not enhance the nerve-mediated erectile response in pathological conditions of nerve injury.
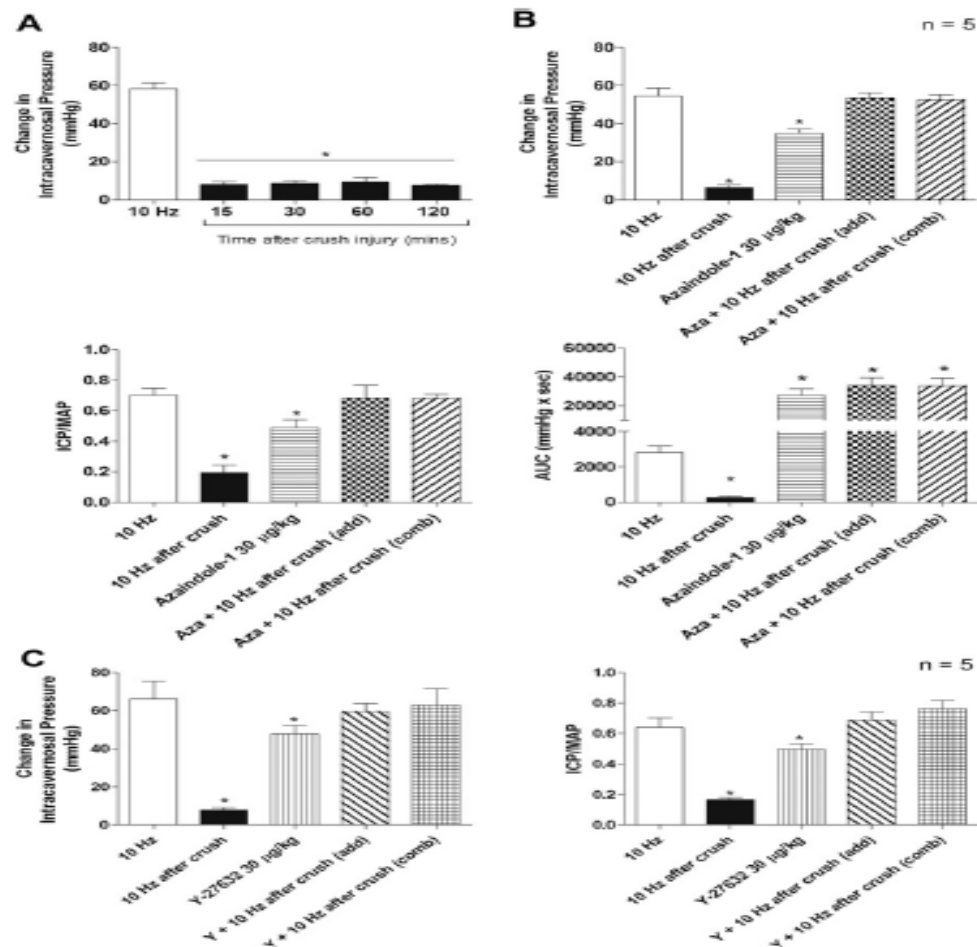
Figure 22. (A) Bar graphs comparing erectile responses before and after nerve crush injury. (B) Bar graphs comparing erectile responses to cavernosal nerve stimulation in the control period (open bar), after nerve crush injury (black bar), intracavernosal injection of azaindole-1 (hatched bar), the algebraic sum of erectile responses to azaindole-1 and nerve stimulation after crush injury (checked bar), and the combined response to simultaneous nerve stimulation after crush injury and intracavernosal injection of azaindole-1 (slanted bar). (C) Bar graphs comparing erectile responses to cavernosal nerve stimulation in the control period (open bar), after nerve crush injury (solid bar), intracavernosal injection of Y-27632 (vertical hatched bar), the algebraic sum of erectile responses to Y-27632 and nerve stimulation after crush injury (slanted bar), and the combined response to simultaneous nerve stimulation after crush injury and intracavernosal injection of Y-27632 at (squared bar). n indicates the number of experiments; * indicates $p < 0.05$ using a one-way ANOVA with a Dunnett post hoc test comparing all values with control. Adapted from Lasker *et al.*, Urology 2013 (108).

IV. Analysis of erectile responses to the sGC stimulator BAY 41-8543 under control and pathophysiological conditions

The effect of intracavernosal injections of BAY 41-8543 on change in ICP, maximum ICP/MAP, MAP, response duration, and AUC was investigated in the anesthetized rat, and these data are summarized in figure 23. The intracavernosal injection of BAY 41-8543 in doses of 0.1–100 µg/kg produced dose-related increases in ICP from 6 ± 1 to 48 ± 2 mmHg. The ICP/MAP ratio at maximum ICP increased in a dose-dependent manner as well (figure 23C) and MAP was decreased 2 ± 1 to 52 ± 4 mmHg in response to intracavernosal injections of BAY 41-8543. The increases in ICP were rapid in onset (10–40 seconds) and the duration of the response was between 53 ± 8 and $2,233 \pm 268$ seconds for doses of 0.1 and 100 µg/kg (figure 23D). The AUC (total erectile response) increased in a dose-dependent manner in response to intracavernosal injections of BAY 41-8543 (figure 23E). These results indicate that the sGC stimulator BAY 41-8543 has significant dose-dependent erectile activity in the anesthetized rat.
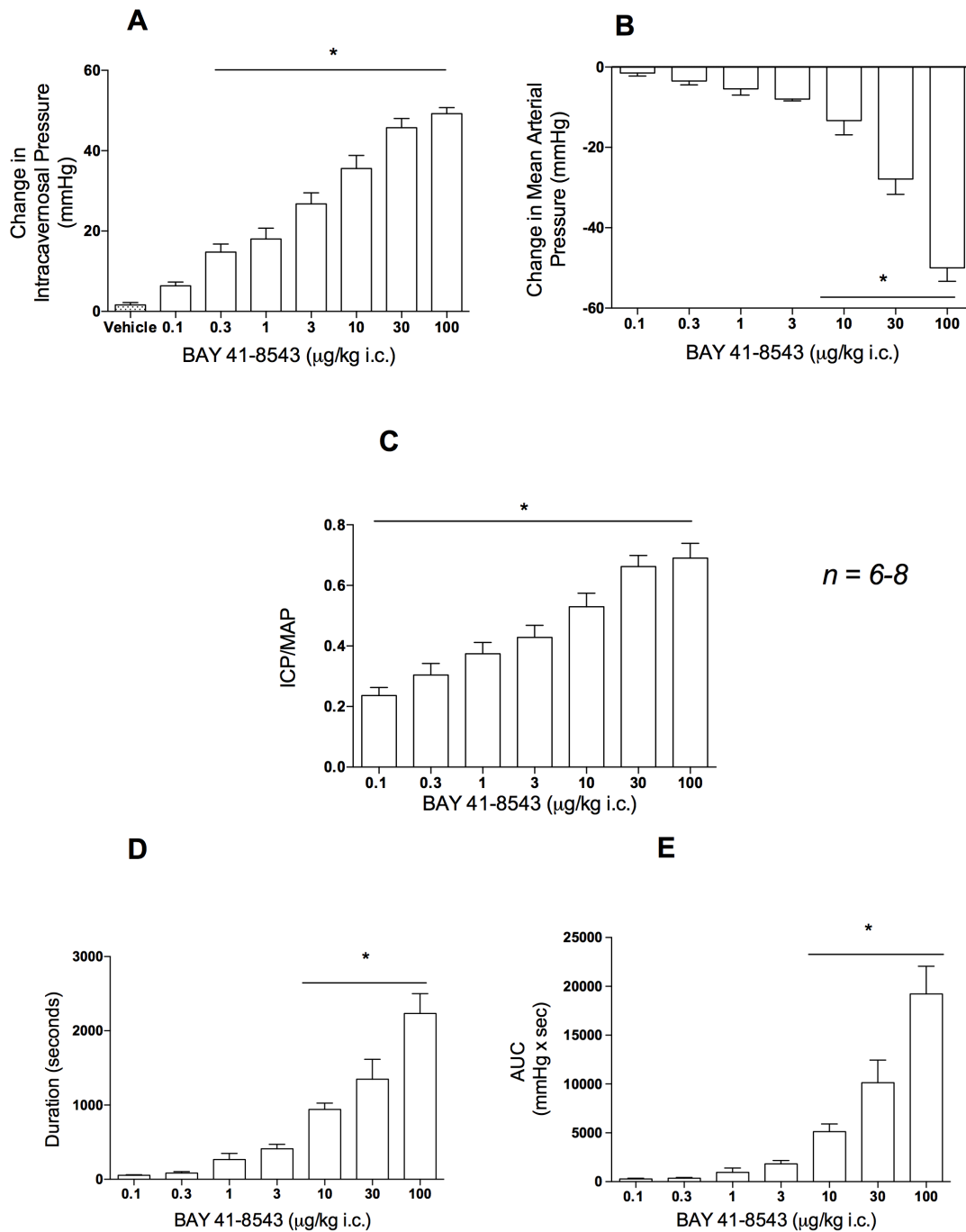
Figure 23. Bar graphs showing erectile responses to intracavernosal injection of a wide range of doses of BAY 41-8543 in the anesthetized rat. n indicates number of experiments, * indicates $p < 0.05$ using one-way ANOVA. Adapted from Lasker *et al.*, J Sex Med 2013 (152).

Erectile responses to intracavernosal injections of BAY 41-8543 and the NO donor sodium nitroprusside were compared and these data are shown in figure 24. The increases in ICP in response to BAY 41-8543 and sodium nitroprusside were compared with doses of the vasodilator agents expressed on a nmol/kg basis to account for differences in molecular weight. These results indicate that changes in ICP in response to intracavernosal injection of sodium nitroprusside are significantly larger than changes in ICP in response to intracavernosal injection of BAY 41-8543 at the higher doses tested (figure 24A). The decreases in MAP in response to intracavernosal injections of BAY 41-8543 and sodium nitroprusside were also compared and show that decreases in MAP after intracavernosal administration of sodium nitroprusside were significantly greater than the systemic vasodilator responses to BAY-41-8543 (figure 24B). However, the duration of the response to intracavernosal injections of BAY 41-8543 was significantly longer when compared with sodium nitroprusside (figure 24C). BAY 41-8543 elicited a significantly greater total erectile response (AUC) as well when compared to sodium nitroprusside (figure 24D). These results indicate that sodium nitroprusside is more potent on a molar basis than BAY 41-8543 in increasing ICP and decreasing MAP, however, responses to BAY 41-8543 were longer in duration and greater in magnitude when the total erectile response (AUC) was taken into account and compared with responses to the NO donor sodium nitroprusside.
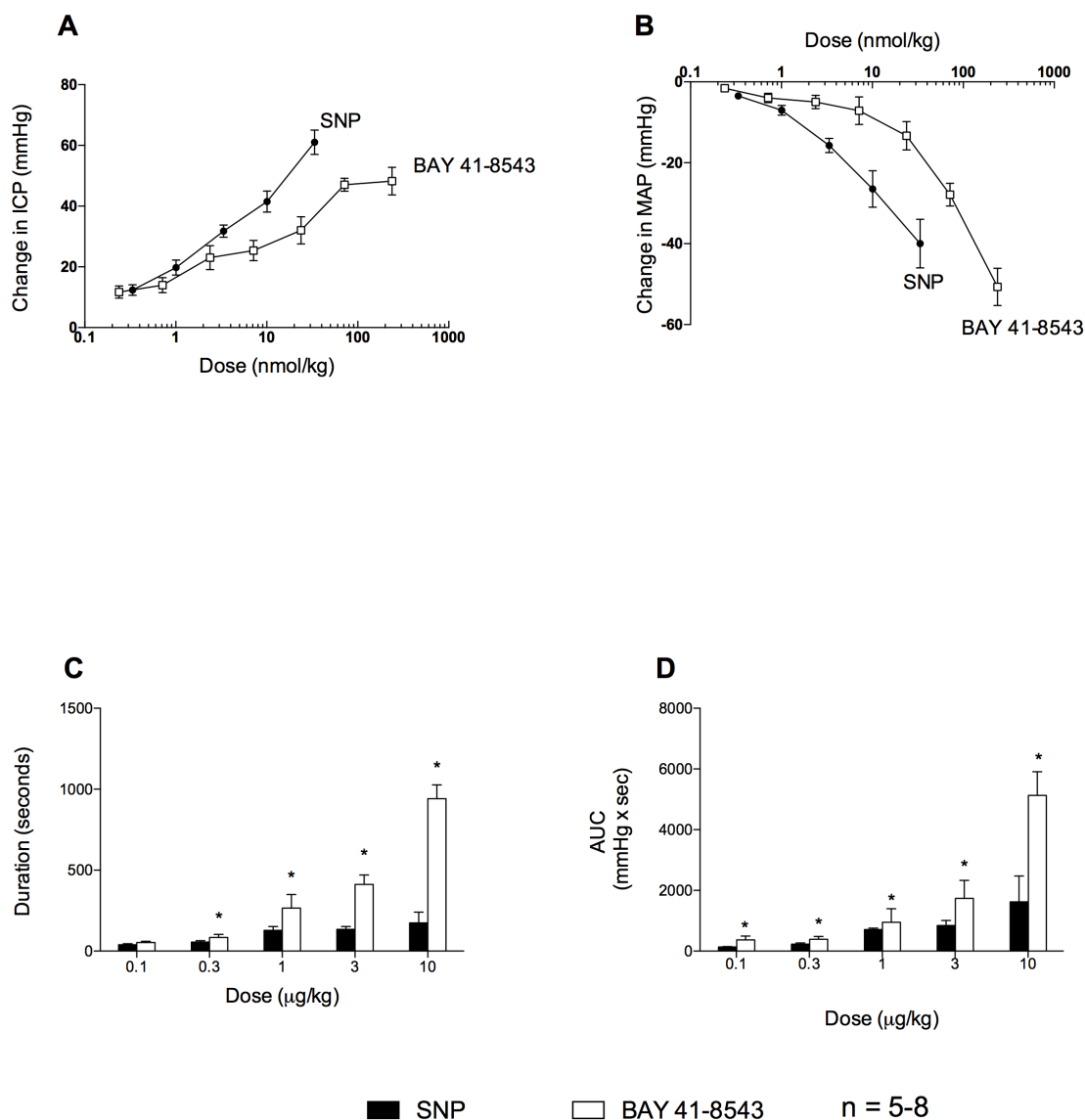
Figure 24. Line graphs comparing erectile responses to intracavernosal injection of BAY 41-8543 and sodium nitroprusside (A & B). Bar graphs comparing the duration (C) and AUC (D) for erectile responses to intracavernosal injections of BAY 41-8543 and sodium nitroprusside. n indicates number of experiments, * indicates $p < 0.05$ when responses to BAY 41-8543 and sodium nitroprusside are compared. Adapted from Lasker *et al.*, J Sex Med 2013 (152).

In order to investigate the interaction between BAY 41-8543 and exogenously administered NO, the erectile response to an intracavernosal injection of a low dose of sodium nitroprusside (0.01 µg/kg) and BAY 41-8543 (0.3 µg/kg) were investigated. In these experiments sodium nitroprusside and BAY 41-8543 were injected separately and then together in a single injection. The intracavernosal injection of sodium nitroprusside (0.01 µg/kg) and BAY 41-8543 (0.3 µg/kg) in separate injections produced increases in ICP of 6 ± 2 and 9 ± 1 mmHg, respectively. The increase in ICP (24 ± 1 mmHg) and AUC in response to the simultaneous injection of BAY 41-8543 (0.3 µg/kg) and sodium nitroprusside (0.01 µg/kg) were significantly greater than the algebraic sum of the individual responses to BAY 41-8543 and sodium nitroprusside (14 ± 2 mmHg) (figure 25A). All intracavernosal injections for these experiments were made in a total fixed volume of 200 µl.

To investigate the interaction between BAY 41-8543 and endogenously released NO, the cavernosal nerve was stimulated at a low frequency (2 Hz) and then nerve stimulation was repeated with simultaneous intracavernosal injection of BAY 41-8543 (0.1 µg/kg). The intracavernosal injection of BAY 41-8543 during cavernosal nerve stimulation produced a significantly larger increase in ICP (38 ± 9 mmHg) and AUC than the algebraic sum of the response to the separate interventions (23 ± 4 mmHg) (figure 25B). The results of these experiments indicate a synergistic interaction between BAY 41-8543 and exogenously
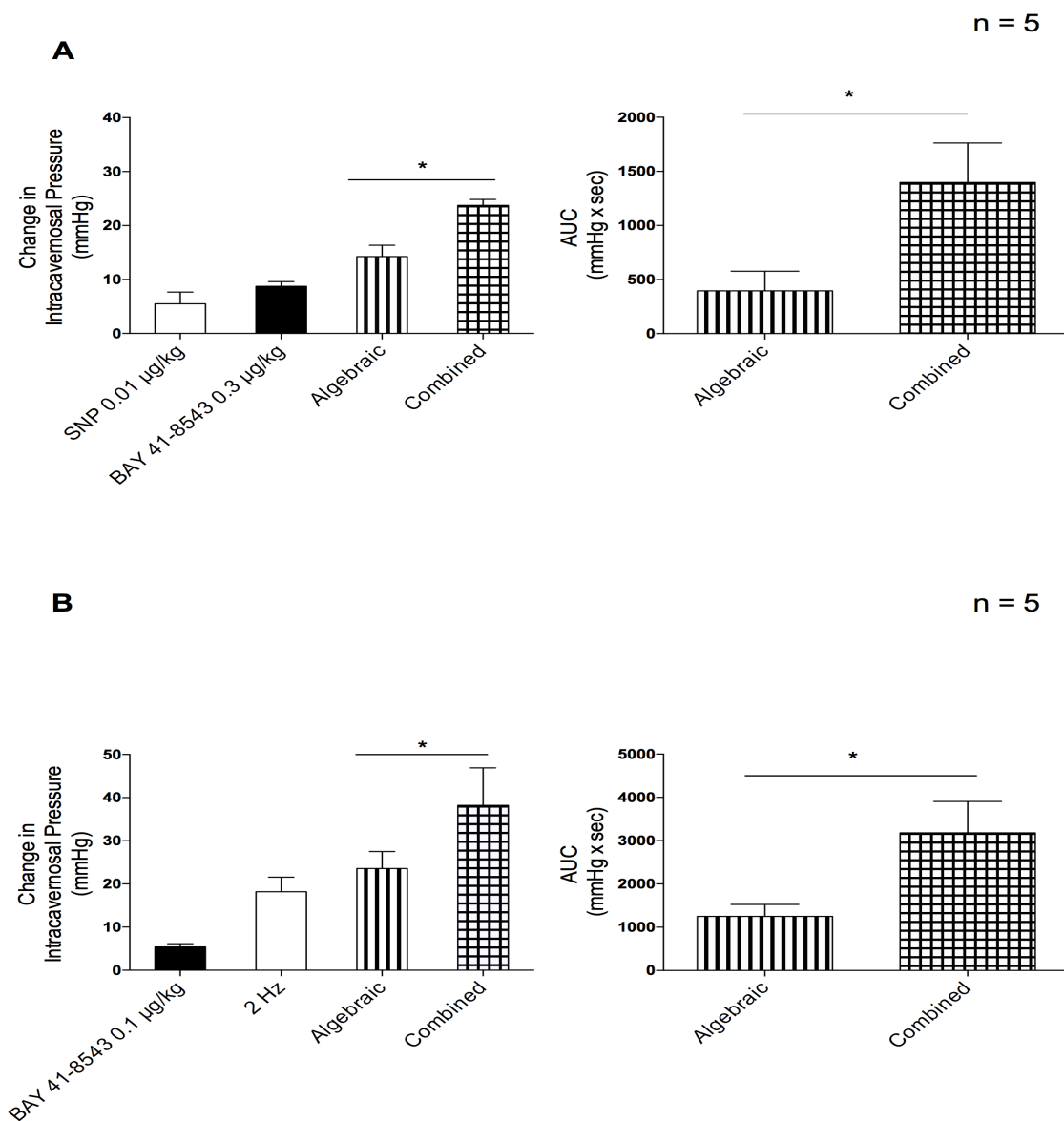
Figure 25. (A) Bar graphs comparing the summation of changes in ICP and AUC for intracavernosal injection with BAY 41-8543 and sodium nitroprusside given separately and then as a combined injection. (B) Bar graphs comparing the summation of changes in ICP and AUC for intracavernosal injection with BAY 41-8543 and cavernosal nerve stimulation at 2 Hz given as two separate interventions and then performed simultaneously. n indicates number of experiments, * indicates $p < 0.05$. Adapted from Lasker *et al.,* J Sex Med 2013 (152).

administered NO and between BAY 41-8543 and endogenously released NO.

In order to determine if BAY 41-8543 can restore the response to cavernosal nerve stimulation after muscarinic receptor blockade, the effect of intracavernosal BAY 41-8543 injection and nerve stimulation after administration of atropine was investigated. The intracavernosal injection of BAY 41-8543 (0.1 µg/kg) restored the increase in ICP in response to cavernosal nerve stimulation at 4 Hz to control value in animals treated with atropine (figure 26). The increase in intracavernosal pressure in response to nerve stimulation at 4 Hz was 48 ± 7 mmHg in the control period and after atropine treatment was 24 ± 4 mmHg. The erectile response was restored to 42 ± 4 mmHg by intracavernosal injection of a low dose of BAY 41-8543 providing evidence for a synergistic response between NO released from NANC nerves (which are unaffected by atropine administration) and the sGC stimulator (figure 26). These data show that BAY 41-8543 is capable of restoring erectile responses to control value following muscarinic receptor blockade in the rat.
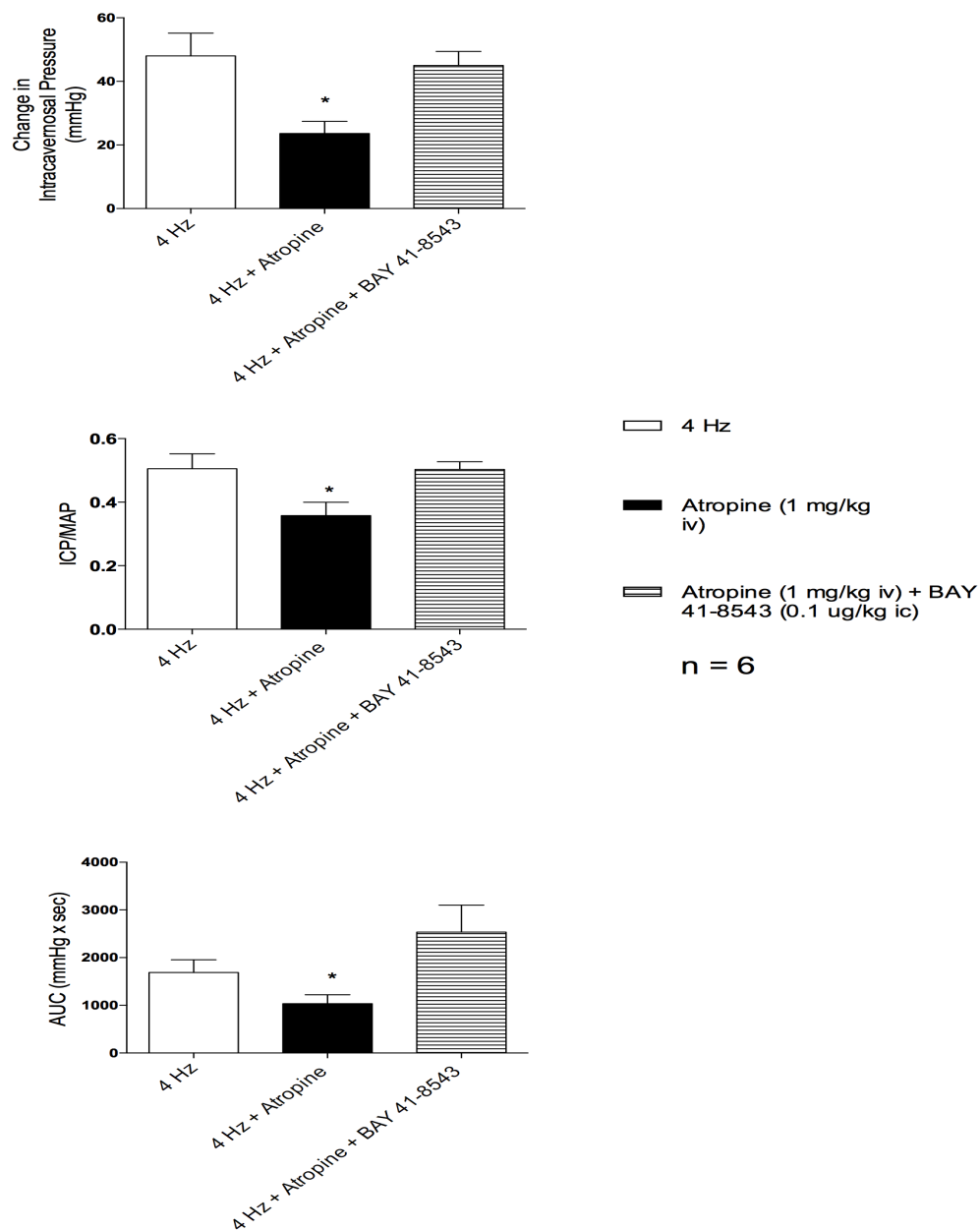
Figure 26. Bar graphs comparing change in ICP (A), ICP/MAP (B), and AUC (C) in response to cavernosal nerve stimulation at 4 Hz before and after administration of atropine 1 mg/kg IV and intracavernosal injection of BAY 41-8543 0.1 µg/kg. n indicates number of experiments, * indicates $p < 0.05$ when compared to response at 4 Hz. Adapted from Lasker *et al.*, J Sex Med 2013 (152).

The effect of intracavernosal injection of BAY 41-8543 on the increase in ICP in response to cavernosal nerve stimulation following nerve crush injury was investigated and these data are summarized in figure 27. Acute cavernosal nerve injury induced by forcep crush reduced the erectile response to cavernosal nerve stimulation at 10 Hz by 83%. A record from an experiment showing the effect of nerve crush injury and of intracavernosal injections of BAY 41-8543 on the attenuated response to cavernosal nerve stimulation is shown in figure 27A. The response to cavernosal nerve stimulation at 10 Hz did not recover spontaneously over the 4-hour period experiments were carried out (n = 3) (figure 27B). The response to cavernosal nerve stimulation was restored to control value by intracavernosal injection of BAY 41-8543 1 µg/kg during cavernosal nerve stimulation (figure 27A, C–E). The experimental record and data obtained clearly demonstrate that the simultaneous administration of BAY 41-8543 with nerve stimulation following crush injury is greater than the additive effect of the interventions applied separately. The results with atropine and cavernosal nerve crush injury can be interpreted to suggest that the synergistic relationship between BAY 41-8543 and endogenous NO may have a beneficial effect in the treatment of ED caused by impaired neurotransmission or iatrogenic injury occurring with prostatectomy.
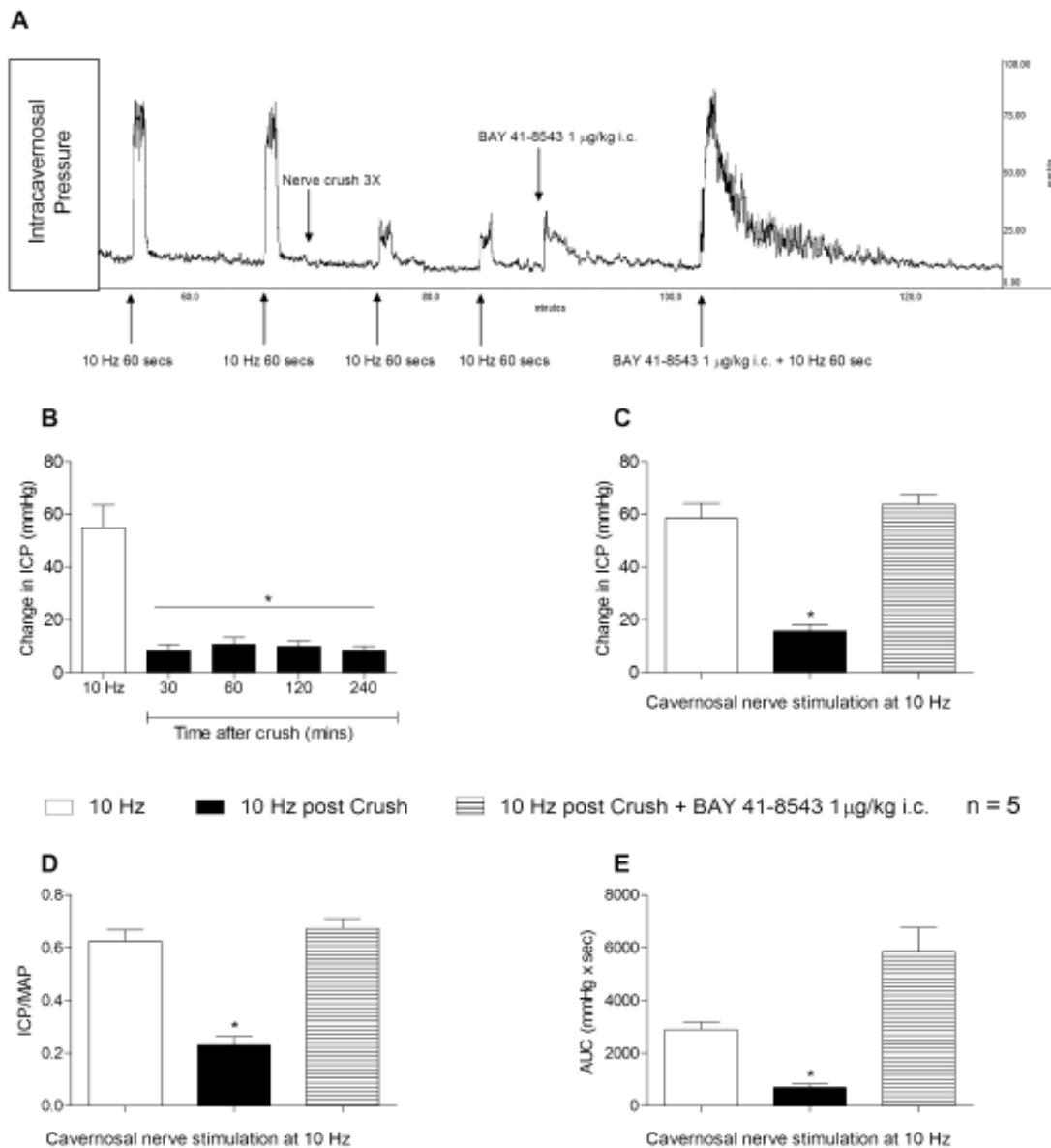
Figure 27. Representative tracing from a cavernosal nerve crush injury experiment in which the increase in ICP in response to nerve stimulation was measured before and after nerve crush and BAY 41-8543 was injected (A). Bar graphs showing the time course of the effect of acute crush of the cavernosal nerve on the responses to cavernosal nerve stimulation (n = 3) (B). Bar graphs erectile responses to cavernosal nerve stimulation in control conditions (open bar), following nerve crush (filled bar), and following nerve crush with simultaneous intracavernosal administration of BAY 41-8543. n indicates number of experiments, * indicates p < 0.05 when compared to control. Adapted from Lasker *et al.*, J Sex Med 2013 (152).

V.   Analysis of erectile responses to the sGC activator BAY 60-2770 under control and pathophysiological conditions

The effect of BAY 60-2770 on erectile function was investigated in the anesthetized rat, and these data are summarized in figure 28. Intracavernosal injection of BAY 60-2770 in doses of 1–300 ng/kg produced dose-related erectile responses (Fig. 28). The increases in ICP in response to intracavernosal injection of BAY 60-2770 were rapid in onset and MAP was only reduced significantly with injections of the higher doses of BAY 60-2770 (Fig. 28). These data indicate that the sGC activator BAY 60-2770 has very potent erectile activity and produces only small decreases in MAP at the higher doses injected intracavernosally in the anesthetized rat. A comparison of the effects of intracavernosal injection of BAY 60-2770 on ICP and of IV injection of BAY 60-2770 on MAP is shown in Fig. 28, bottom right. These data indicate that BAY 60-2770 is far more potent in its ability to increase ICP than in its ability to produce vasodilation in the systemic vascular bed and decrease MAP in the rat.
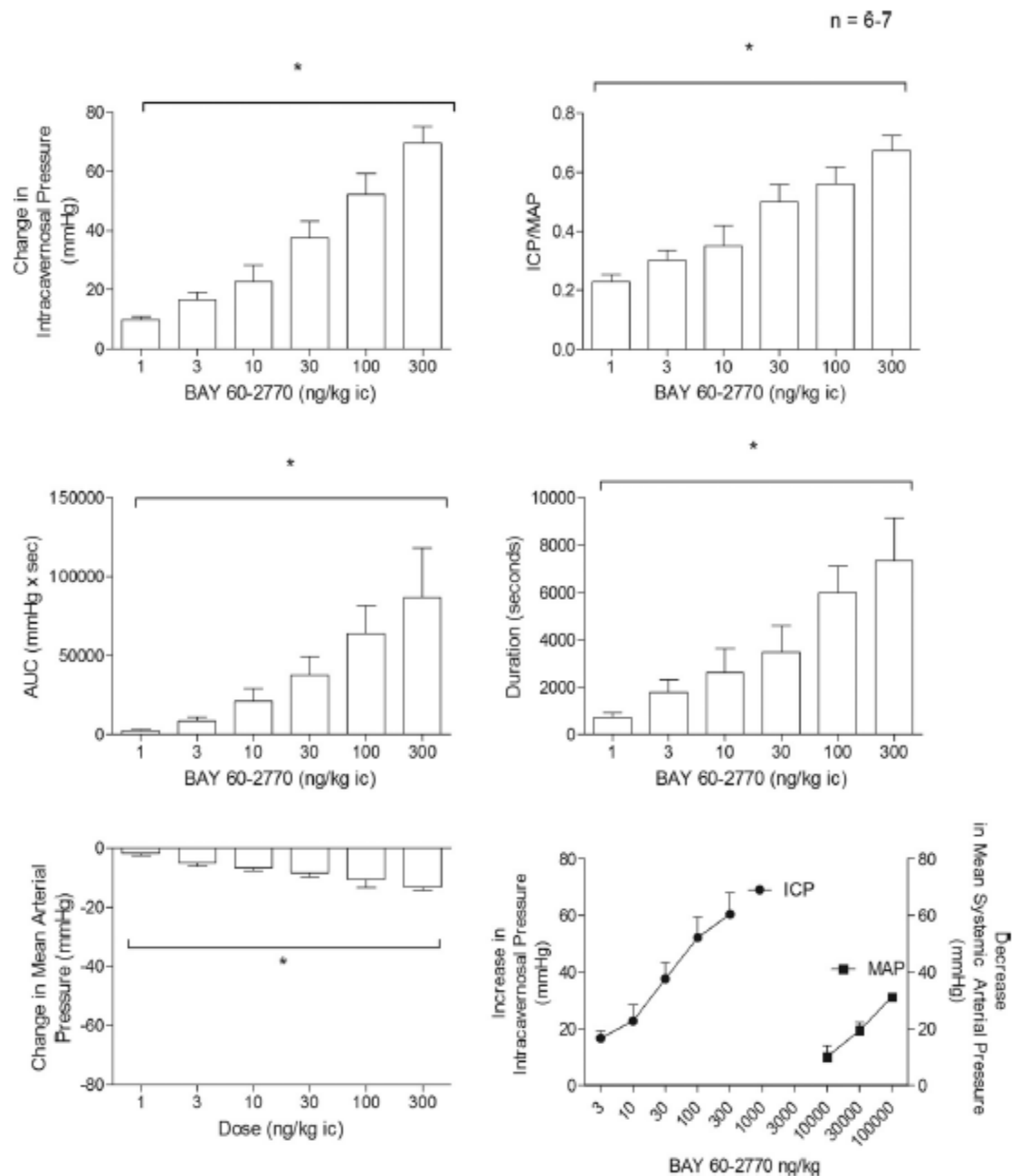
Figure 28. Bar graphs showing the dose-dependent erectile and systemic vasodilator activity of BAY 60-2770 in the anesthetized rat. Line graph in the bottom right panel shows a comparison of responses to intracavernosal injections and intravenous injections of BAY 60-2770 on intracavernosal and mean systemic arterial pressure. n indicates number of experiments, * indicates $p < 0.05$ with ANOVA. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).

ODQ is an agent that has been shown to inhibit NO-mediated activation of sGC by oxidizing the heme iron on the beta subunit of the smooth muscle enzyme (128, 134). sGC activators like BAY 60-2770 can increase the catalytic activity of oxidized or heme-free sGC as reported by Schmidt *et al.* (154). The effect of ODQ treatment on the erectile response to BAY 60-2770 was investigated, and these data are shown in figure 29. After treatment with ODQ, 2 mg/kg, the increases in ICP, ICP/MAP, and AUC in response to intracavernosal injections of BAY 60-2770 (1-10 ng/kg) were enhanced significantly when the erectile responses to intracavernosal injection of the NO donor sodium nitroprusside were attenuated (Fig. 29 & Fig. 16, respectively). Similarly, in contrast to the effect of ODQ treatment on erectile responses to intracavernosal injection of the sGC activator BAY 60-2770, the increases in ICP, ICP/MAP, and AUC in response to intracavernosal injection of the reduced heme-dependent sGC stimulator BAY 41-8543 were significantly reduced by ODQ treatment (Fig. 30). The comparison of dose-response data obtained for intracavernosal injections of BAY 60-2770 and BAY 41-8543 show that the potency of the sGC activator BAY 60-2770 is approximately 1,000-fold greater than the erectile activity of the sGC stimulator BAY 41-8543 under control conditions (Fig. 31).
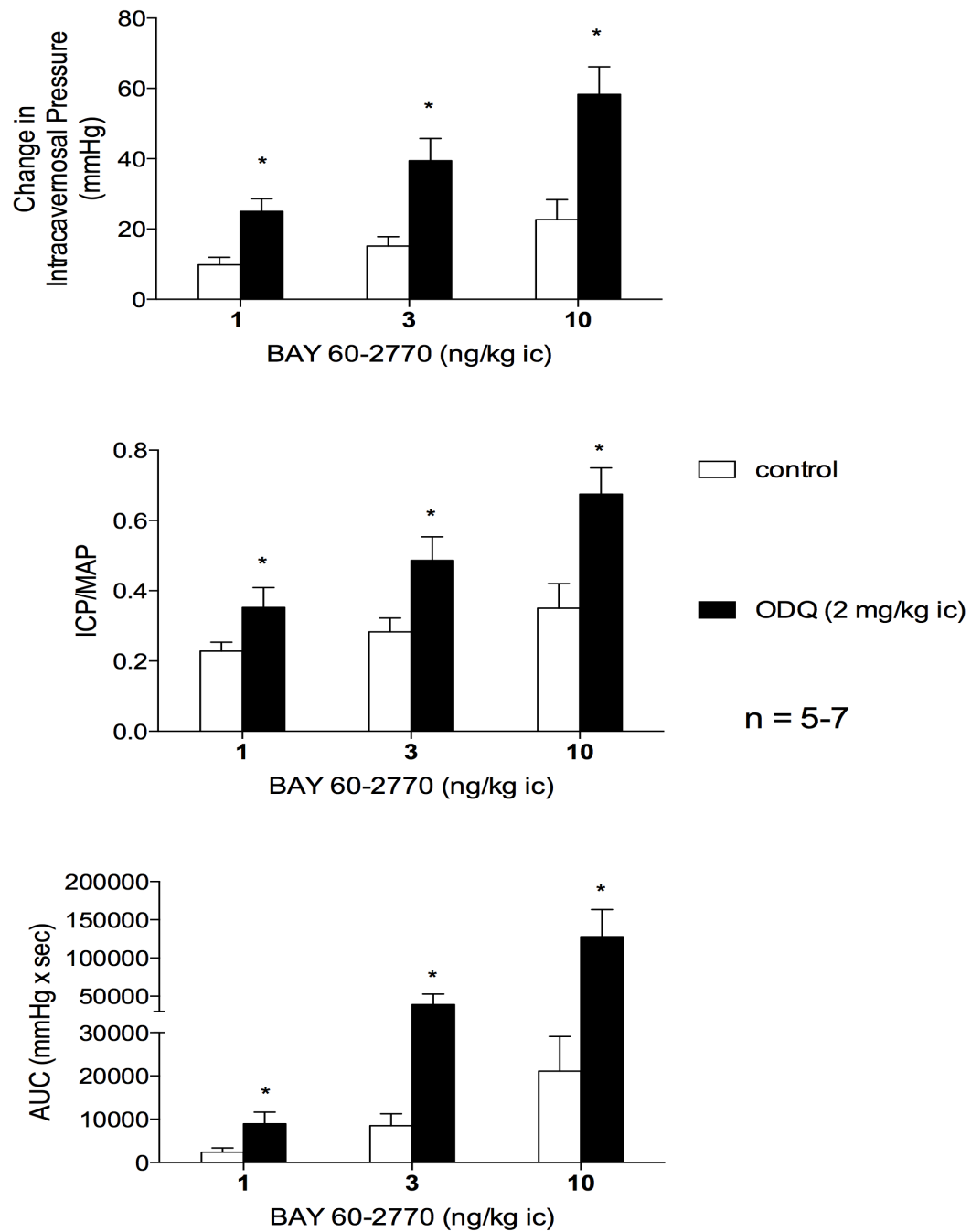
Figure 29. Bar graphs showing the effect of treatment with ODQ (2 mg/kg) on the increases in ICP, ICP/MAP, and AUC in response to intracavernosal injections of BAY 60-2770. n indicates number of experiments, * indicates $p < 0.05$ using paired t-test. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).
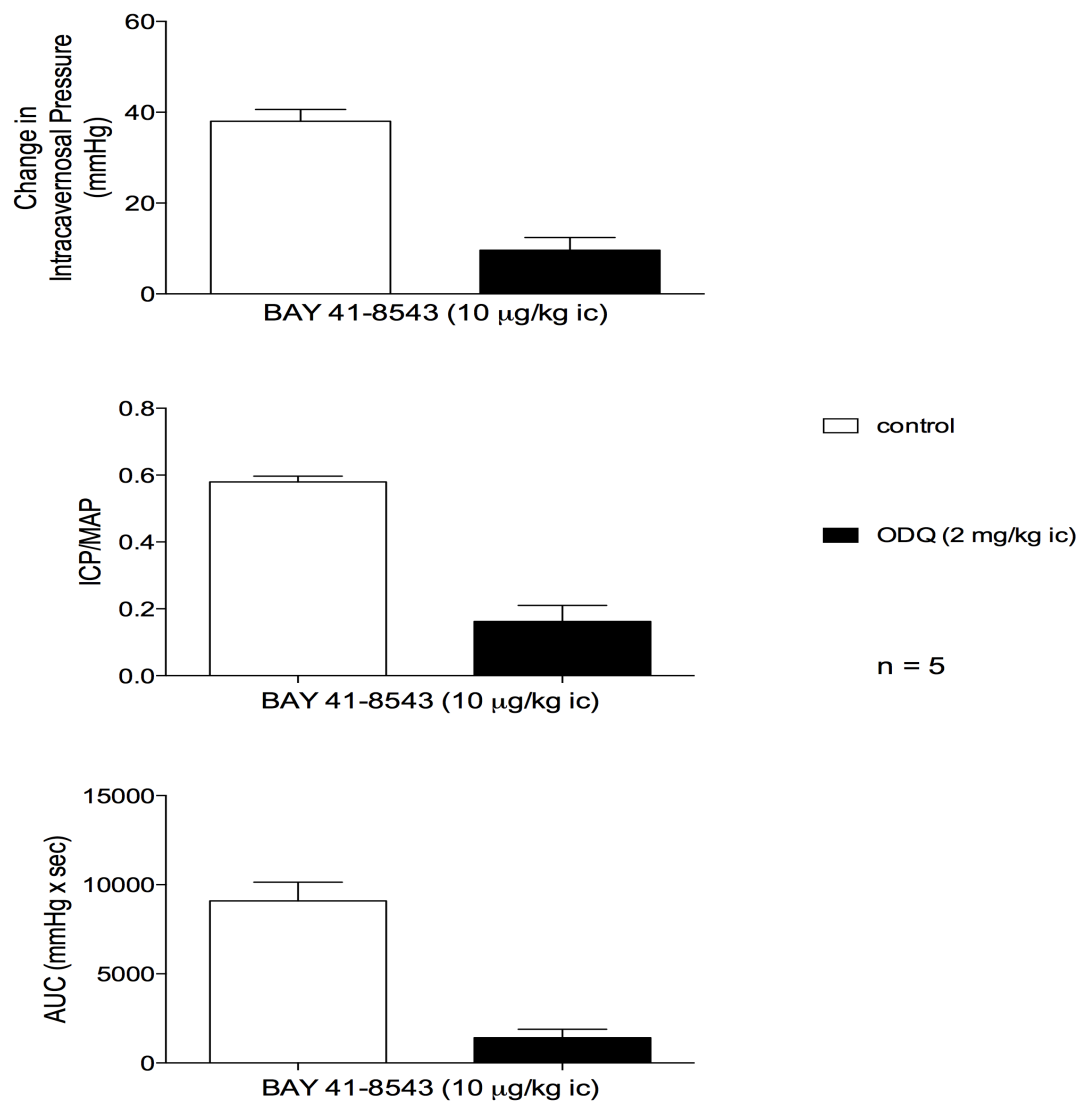
Figure 30. Bar graphs comparing the effect of ODQ treatment (2 mg/kg) on the changes in ICP, ICP/MAP, and AUC in response to intracavernosal injection of BAY 41-8543. n indicates number of experiments, * indicates $p < 0.05$ using paired t-test. Adapted from Lasker *et al.,* Am J Physiol Heart Circ Physiol 2013 (153).
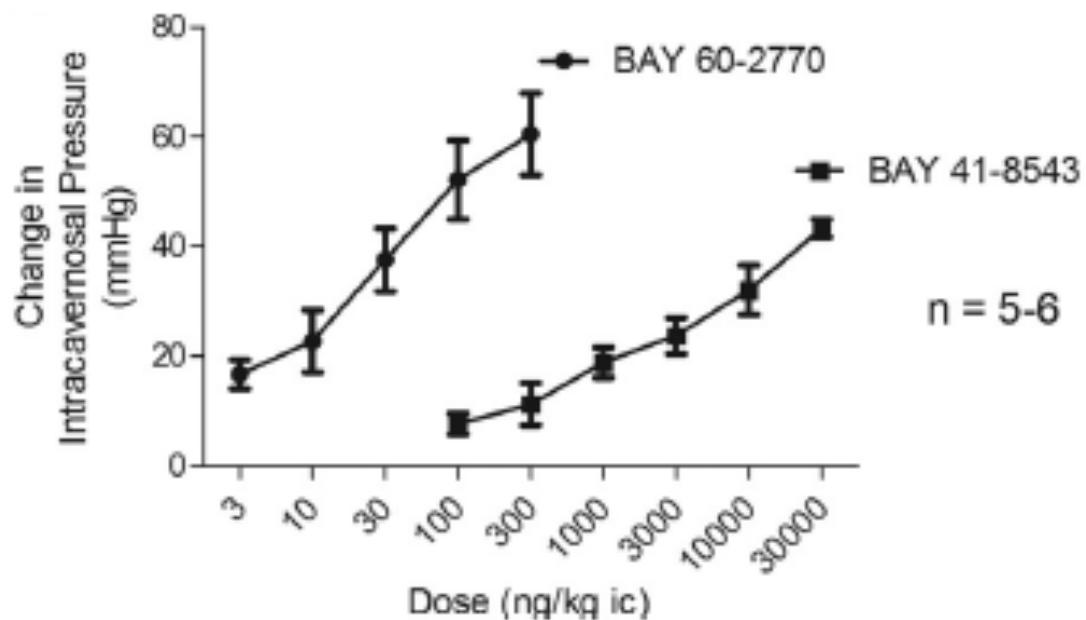
Figure 31. Line graphs comparing change in ICP in response to intracavernosal injections of BAY 60-2770 and BAY 41-8543. n indicates number of experiments; error bars indicate standard error of the mean value. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).

The effect of cavernosal nerve crush injury on the response to BAY 60-2770 was investigated, and the data are summarized in figure 32. Acute (n = 4) and chronic (n = 2) nerve crush injury caused a significant decrease in erectile responses to cavernosal nerve stimulation. The increases in ICP, ICP/MAP, or AUC in response to intracavernosal injections of BAY 60-2770 after nerve crush injury were not different from responses obtained in control animals (Fig. 32).

The effect of the NOS inhibitor L -NAME on the response to BAY 60-2770 was investigated and treatment with L -NAME 50 mg/kg IV significantly attenuated erectile responses to cavernosal nerve stimulation at all stimulation frequencies tested (Fig. 15). The intracavernosal injection of 100 ng/kg BAY 60-2770 in animals treated with L -NAME produced erectile responses that were no different from the responses recorded from intracavernosal injection of the same dose in control animals (Fig. 33). These data indicate that the erectile response to BAY 60-2770 is independent of endogenous NO release in the rat penis.
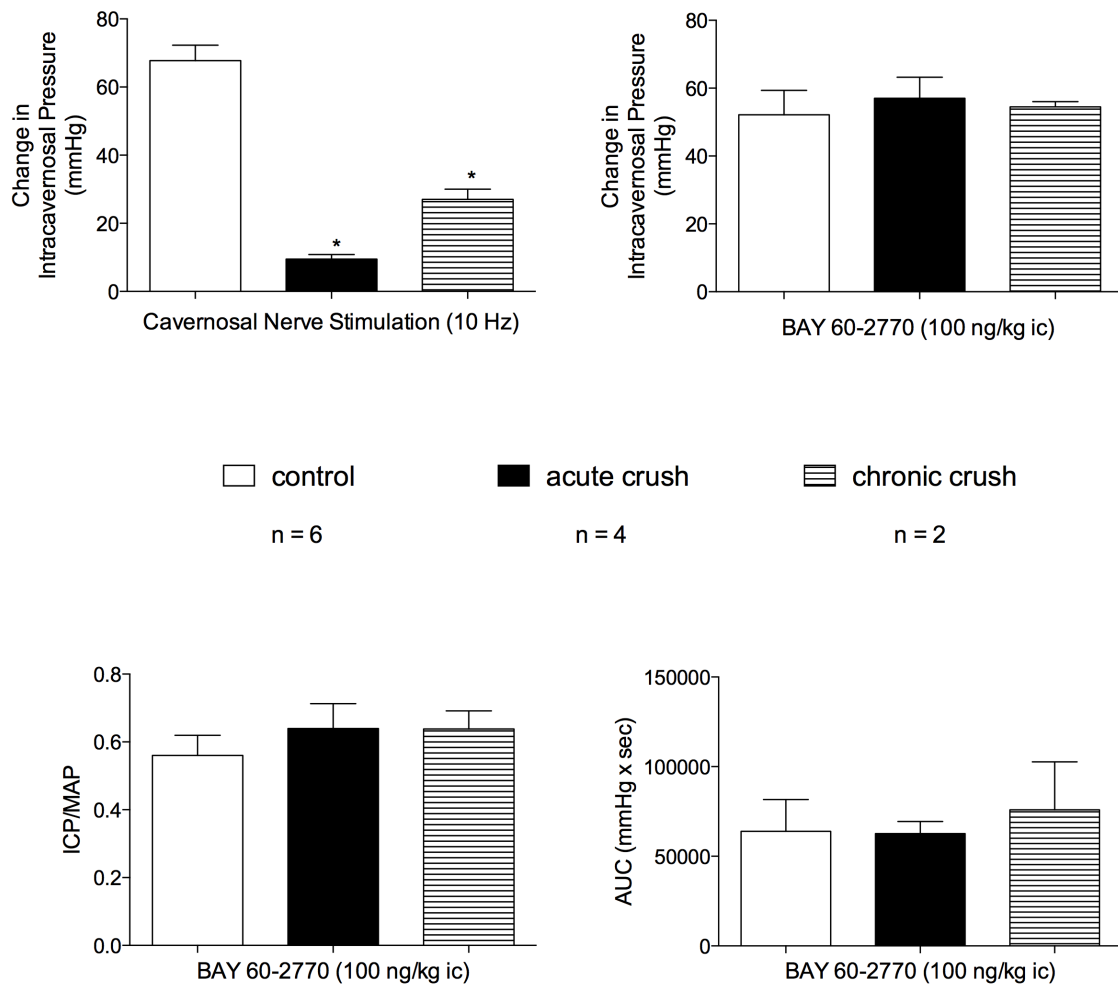
Figure 32. Bar graphs illustrating the effect of cavernosal nerve crush injury (solid bars) on the change in ICP in response to cavernosal nerve stimulation at 10 Hz and the effect of acute (n = 4) and chronic (n = 2) cavernosal nerve crush injury on the responses to intracavernosal injection of BAY 60-2770 (100 ng/kg). n indicates number of experiments, * indicates $p < 0.05$ when compared to control. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).
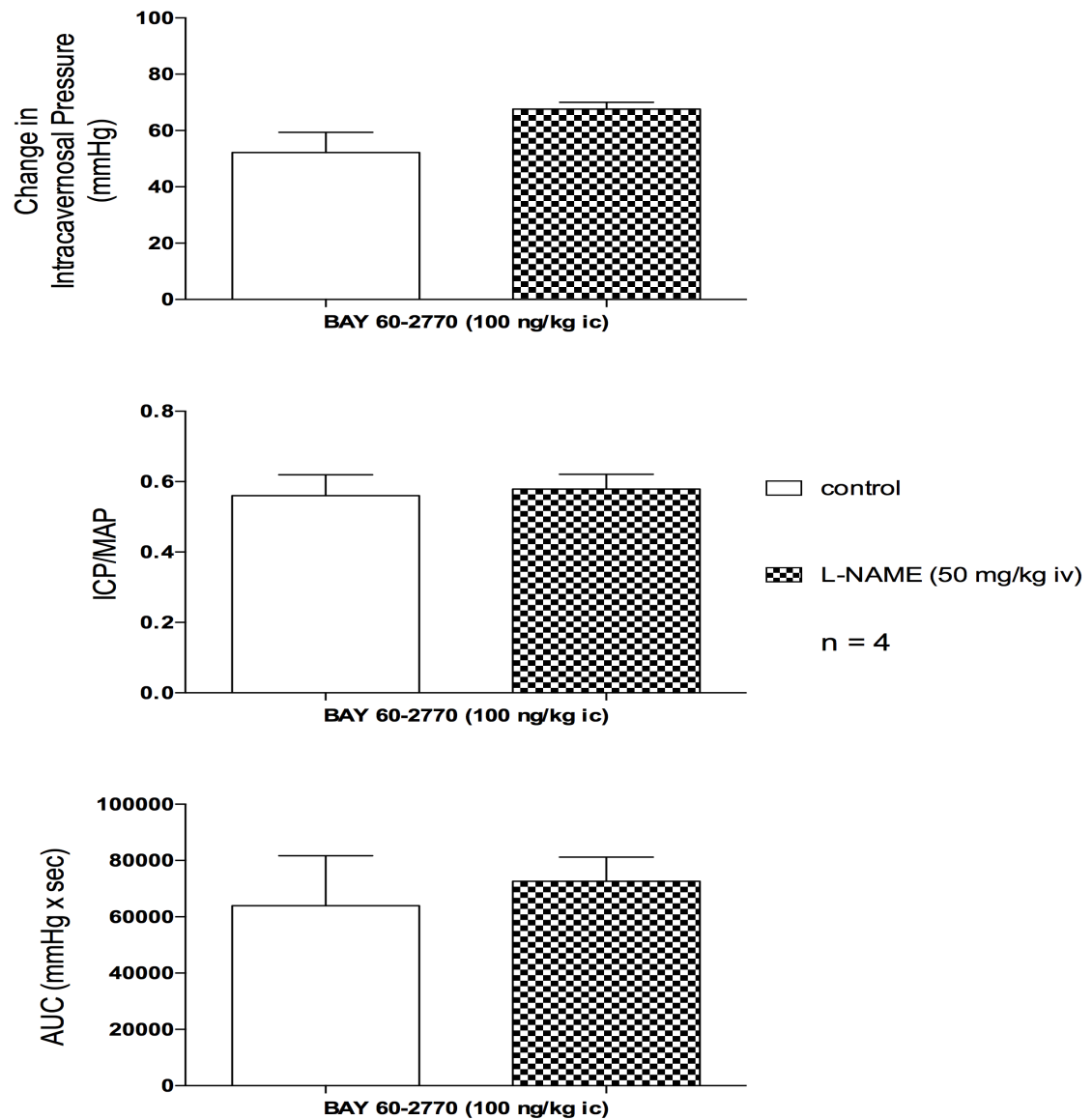
Figure 33. Bar graphs showing the effect of L-NAME treatment on the change in ICP, ICP/MAP, and AUC in response to intracavernosal injection of BAY 60-2770 100 ng/kg. n indicates number of experiments; error bars indicate standard error of the mean value. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).

The potential role of phosphodiesterase inhibition in contributing to the erectile response to intracavernosal injections of BAY 60-2770 was investigated, and these results are presented in figure 34. The administration of the PDE-5 inhibitor avanafil increased the AUC (total erectile response) to the NO donor sodium nitroprusside whereas a dose of BAY 60-2770 which had the same systemic vasodepressor effect as the dose of avanafil used had no significant effect on the erectile response to sodium nitroprusside (Fig. 34A). These data indicate that BAY 60-2770 had no apparent effect on response duration whereas the PDE-5 inhibitor avanafil increased the AUC of the response to the NO donor. Avanafil increased the AUC of the response to BAY 60-2770 under control conditions and after treatment with ODQ (Fig. 34B). Taken together, these data provide evidence that increased cGMP concentration mediates the erectile response to BAY 60-2770 when sGC is normally reduced or oxidized by ODQ and suggest that BAY 60-2770 does not affect the degradation of cGMP through phosphodiesterase inhibition.
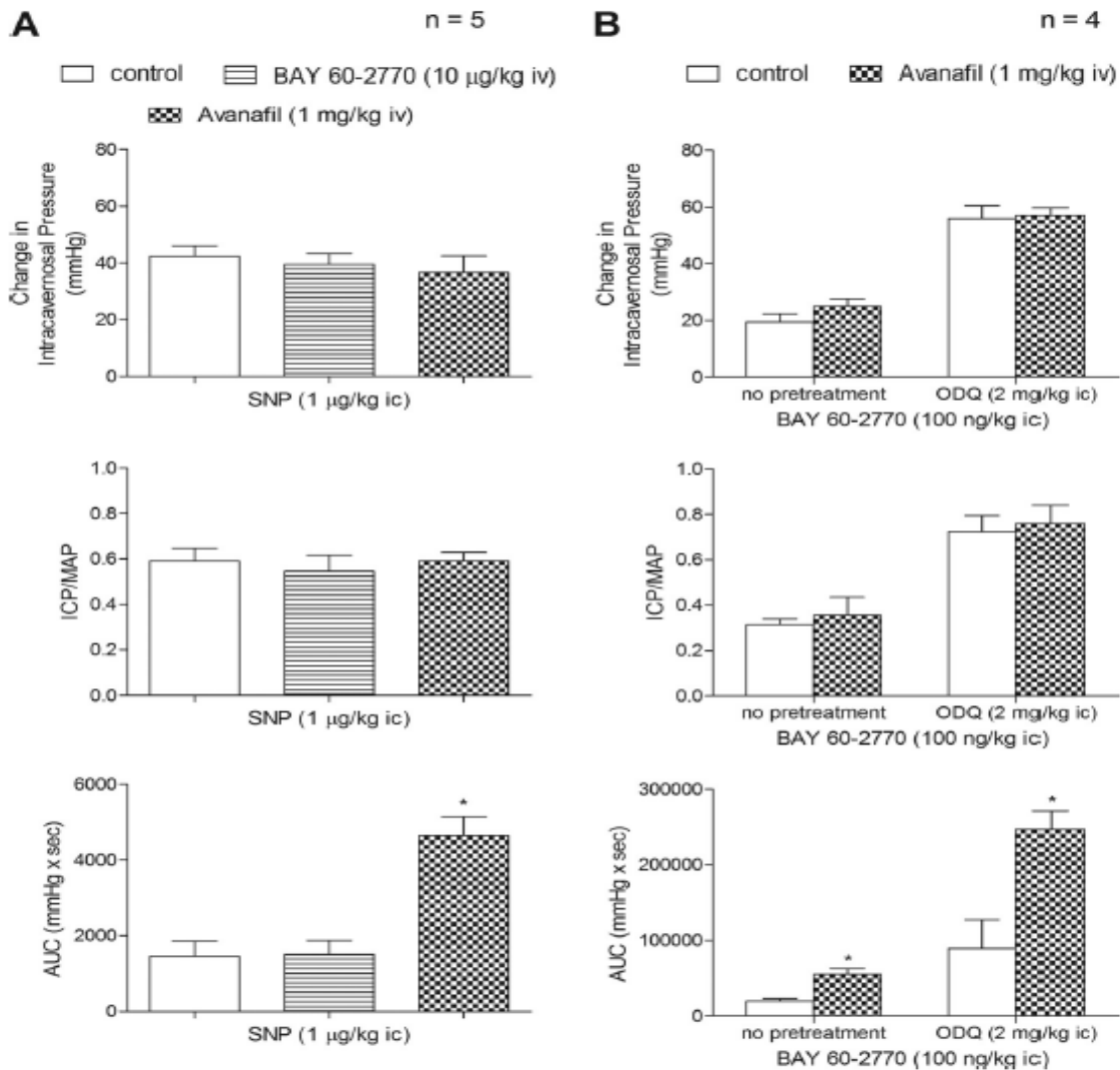
Figure 34. Bar graphs showing the effect of treatment with the PDE-5 inhibitor avanafil or BAY 60-2770 on erectile responses to intracavernosal injections of the NO donor sodium nitroprusside (A). Bar graphs showing the effect of treatment with avanafil, ODQ or combination treatment of avanafil and ODQ on erectile responses to intracavernosal injections of BAY 60-2770 (B). n indicates number of experiments, * indicates $p < 0.05$ when compared to control. Adapted from Lasker *et al.,* Am J Physiol Heart Circ Physiol 2013 (153).

DISCUSSION

I.   <u>Effect of muscarinic receptor antagonism on the response to cavernosal nerve stimulation</u>

NO is the principal mediator of penile erection and during the erectile response NO formed by nNOS is released from NANC nerves innervating the corpora cavernosum as well as cholinergic nerves where ACh and nNOS may be co-localized (38). Although the role of NO released from nerves innervating the corpora cavernosa in mediating penile erection is well-established, the role of muscarinic receptor activation by cholinergic nerve-mediated release of ACh is less well established (17). The contribution of muscarinic receptor stimulation in mediating the erectile response to cavernosal nerve stimulation in the anesthetized rat was examined by investigating the effect of atropine on erectile responses. The increase in ICP, ICP/MAP, and AUC in response to cavernosal nerve stimulation was reduced significantly at stimulus frequencies of 2, 4, 8, 10, and 16 Hz after treatment with the muscarinic receptor antagonist in a dose of 1 mg/kg IV. In these experiments, the increase in ICP in response to intracavernosal injection of ACh was significantly reduced when the erectile responses to intracavernosal injections of sodium nitroprusside were not

changed. NO generated by eNOS in response to activation of muscarinic receptors by ACh released from cholinergic nerve terminals mediates approximately 30% of the erectile response in the present study. The hypothesis that release of NO from cavernosal nerve terminals has an important role in the erectile response is based on studies showing that inhibitors of nNOS have major inhibitory effects on erectile responses to cavernosal nerve stimulation in anesthetized animals (155, 156). Our data with 7-NI suggest that nNOS activity in the NANC nerves mediates a significant component of the erectile response to cavernosal nerve stimulation in the anesthetized rat. In agreement with previous studies (31), our data suggests that after treatment with the nonselective NOS inhibitor, L-NAME, erectile responses to cavernosal nerve stimulation are decreased by approximately 90%.

The data with ACh and sodium nitroprusside establish the efficacy and selectivity of the muscarinic receptor blockade produced by atropine. These results suggest that the response to cavernosal nerve stimulation is mediated by the release of NO and ACh from cavernosal nerves and the endothelium. In addition, the fall in MAP in response to IV injections of sodium nitroprusside were not reduced after administration of atropine. The decrease in MAP in response to IV injection of ACh was associated with no significant change in cardiac output or heart rate when values were measured at the time of the peak decrease in MAP. This indicates that the decrease in MAP in response to IV injection of ACh was

caused by a cholinergically mediated decrease in systemic vascular resistance and that ACh has potent vasodilator activity in the penile and systemic vascular beds.

The mechanism by which ACh induces vasodilation in the penis was investigated in experiments with the nonselective NOS inhibitor L-NAME. Following treatment with L-NAME, the increase in ICP in response to intracavernosal injection of ACh was significantly attenuated suggesting that the release of NO from the endothelium in the corpora cavernosa is involved in mediating the response. These results suggest that muscarinic receptor activation plays a significant role in mediating the erectile response to cavernosal nerve stimulation in the rat and provide evidence that the release of NO in response to cholinergic signaling occurs through activation of NOS.

The present results are consistent with results in the literature showing that the erectile response to cavernosal nerve stimulation is reduced in the monkey dog and rat by the administration of atropine (45, 46, 51, 157). Senbel *et al.* reported that the inhibitory effect of atropine on erectile responses in the rat was both dose- and stimulation frequency-dependent with inhibition of 5–25% at the 1.5 mg/kg dose of atropine and 25–55% at the 10 mg/kg dose of atropine (157). The present data support the concept that muscarinic receptor activation plays a significant role in mediating the erectile response to cavernosal nerve

stimulation in the rat.

The present results are not in agreement with the studies in the literature showing that the response to nerve stimulation in the dog and rat is not significantly altered by atropine (44, 47). Dail *et al.* reported that atropine had no effect on the response to cavernosal nerve stimulation in the rat, however, after cavernosal nerve ablation, erectile responses to pelvic nerve stimulation were attenuated by atropine (47). The explanation for the difference in results is uncertain, but does not involve specificity of the inhibitory effect of atropine for muscarinic receptors which has been suggested by some authors, because erectile responses to intracavernosal injections of ACh were attenuated at a time when responses to sodium nitroprusside were not altered in the present study (17).

To summarize, these data suggest that the activation of muscarinic receptors mediate approximately 30% of the increase in ICP in response to cavernosal nerve stimulation and indicate that the cavernosal nerves contain NO and ACh releasing terminals, although experiments with larger doses of atropine are needed to establish and quantify this effect. The erectile response to intracavernosal injection of ACh was attenuated by L-NAME and atropine, providing evidence that the response to ACh was mediated by the generation of NO from NOS following the activation of muscarinic receptors by ACh in the

corpora cavernosa of the rat.

II.   <u>Effect of the soluble guanylate cyclase inhibitor ODQ on erectile responses in the rat</u>

ODQ has been reported to inhibit the response to NO by oxidizing the heme iron on the beta subunit of sGC (134). ODQ has also been shown to inhibit NO-mediated sGC activation in a large number of isolated tissue studies and to inhibit vasodilator responses to NO donors in a few in vivo studies (126, 130-134).   NO binds to the reduced form of sGC and increases the conversion of GTP to cGMP, promoting vascular and cavernosal smooth muscle relaxation and penile erection. When sGC is oxidized by ODQ, the enzyme becomes insensitive to NO and vasorelaxation and the subsequent erectile response are inhibited. Our results provide evidence that administration of ODQ attenuates erectile responses to cavernosal nerve stimulation and to intracavernosal injection of the NO donors DEA/NO and sodium nitroprusside, providing support for the hypothesis that the sGC inhibitor oxidizes the heme moiety of the heterodimeric enzyme (134). Therefore, these data suggest that ODQ may be used in vivo acutely to model pathophysiological conditions of prolonged oxidative stress.

The effect of ODQ on erectile responses to isoproterenol and imatinib was investigated. The observation that erectile responses to the beta-adrenergic

receptor agonist isoproterenol or an agent which inhibits tyrosine kinase signaling

are not altered by ODQ in a dose that attenuated responses to the NO donors

sodium nitroprusside and DEA/NO suggests that the actions of ODQ are

selective for NO and are not nonspecific or off-target. The observation that

methemoglobin levels were not significantly altered suggests the effect of ODQ

on the heme iron of sGC is selective and the iron of hemoglobin is not

substantially oxidized by the dose of ODQ used in the present study. The effect

of ODQ treatment on the response to electrical stimulation of the cavernosal

nerves was investigated. The results of these experiments show that erectile

responses to cavernosal nerve stimulation at 2-16 Hz are attenuated and indicate

that ODQ treatment decreases the catalytic activity of sGC in penile vascular and

corporal smooth muscle to neurogenically released NO. These data taken

together demonstrate that the in vivo use of ODQ is selective for the beta subunit

of sGC, has little off-target effect on other heme containing proteins, and

attenuates erectile responses to exogenous and endogenous NO in the

anesthetized rat.

III.   Role of Rho-kinase in NO-mediated erectile responses

Azaindole-1, the selective Rho-kinase inhibitor, had potent long-lasting

erectile activity that was independent of NANC neurotransmission, muscarinic

receptor or sGC activation and possessed erectile activity that was not affected

by acute cavernosal nerve injury. The present results show that intracavernosal injections of azaindole-1 produced changes in ICP in the anesthetized rat that were similar to equivalent doses of fasudil, the prototypical Rho-kinase inhibitor, when doses were expressed on a microgram per kilogram basis; however, the AUC and duration of erectile responses were greater for azaindole-1 than for fasudil. Both Rho-kinase inhibitors were less potent than the NO donor sodium nitroprusside, but had a longer duration of action than the nitro-vasodilator and can produce priapism.

The role of NO release from NANC nerves and the endothelium in response to ACh stimulation of muscarinic receptors was investigated in relation to Rho-kinase inhibitors and we found that erectile responses to azaindole-1 and fasudil were not altered by treatment with the nNOS inhibitor 7-NI when administered in combination with the cholinergic antagonist atropine. The administration of 7-NI and atropine together attenuated erectile responses to cavernosal nerve stimulation significantly. These results indicate that the increases in ICP in response to azaindole-1 and fasudil are independent of NO released from the cavernosal nerves or activation of muscarinic receptors in the corpora cavernosa. These data are consistent with studies in the literature showing that erectile responses to Y-27632, the first widely studied Rho-kinase inhibitor, were not modified significantly by pretreatment with the nonselective NOS inhibitor L –NAME (98).

The present results show that increases in ICP in response to the Rho-kinase inhibitors azaindole-1 and Y-27632 are not altered by acute cavernosal nerve crush injury. These results are consistent with a previous study in which corporal relaxation responses to Y-27632 were not modified by inhibition of nonadrenergic, noncholinergic function but are not in agreement with studies in which the relaxation response to Y-27632 was reduced in in vitro cavernosal tissue preparations from diabetic or L-NAME treated animals (158). The reason for the difference in results may involve variation in experimental species (rabbit vs. rodent) and different experimental design (in vitro tissue preparation vs. in vivo administration).

The effect of inhibition of sGC with ODQ on erectile responses was investigated, and after treatment with ODQ, the increase in ICP in response to intracavernosal injection of sodium nitroprusside was reduced by 87%. ODQ had no significant effect on erectile responses to the intracavernosal injection of azaindole-1 or fasudil. These data provide support for the hypothesis that erectile responses to azaindole-1 and fasudil are independent of activation of sGC in rat penile tissue and are consistent with results showing that ODQ did not alter relaxation responses to the Rho-kinase inhibitors H-1152 and Y-27632 in phenylephrine precontracted rat cavernosal tissue strips (159).

The present results and previous studies provide support for the concept that Rho-kinase inhibitors would be useful in the treatment of ED caused by impaired NO release, impaired muscarinic receptor activation, or impaired activation of sGC by NO (104, 160). Penile erection involves a complex interaction between the central nervous system and local mediators (161). Agents or disease processes that impair NO formation, release, or bioavailability and NO-sGC-cGMP signaling induce ED, and agents that improve NO-sGC-cGMP signaling improve erectile function (162, 163). The ability of the Rho-kinase inhibitors to improve erectile responses when NO formation, release, or signaling are impaired or cavernosal nerve injury has occurred suggest that these agents may be useful in the treatment of ED resulting from diverse pathophysiological mechanisms (102, 164).

A number of studies suggest that alteration in Rho-kinase activity is involved in the pathogenesis of a variety of cardiovascular diseases, including those associated with ED (165, 166). Much information has been learned from studies with fasudil, which has been approved for the treatment of cerebral vasospasm in subarachnoid hemorrhage and shown efficacy in the treatment of cerebral ischemia (167). Although fasudil and Y-27632 have been useful in defining the role of Rho-kinase in physiologic and pathophysiologic processes, these agents have nonspecific inhibitory effects on other kinases, such as protein kinase A, which can alter vascular smooth muscle function (107).

Azaindole-1 is a highly selective, orally effective Rho-kinase inhibitor that has little if any effect on a number of the kinases involved in smooth muscle function and has a favorable effect in a number of cardiovascular disorders (168, 169). The results of the present study with azaindole-1 are similar to data with SAR407899, another novel Rho-kinase inhibitor without NO-cGMP dependence that has recently been shown to exert long-lasting erectile responses after oral administration and did not lose efficacy in diabetic or hypertensive animals (158). The results with potent long-lasting Rho-kinase inhibitors, such as azaindole-1 or SAR407899, suggest that these agents may be useful in the treatment of patients with severe ED. However, the potential to induce priapism with the use of long-acting Rho-kinase inhibitors should be considered.

The interaction of Rho-kinase and endogenous NO was also investigated in experiments in which the response to cavernosal nerve stimulation and intracavernosal injection of the Rho-kinase inhibitors were compared when the interventions were administered separately or together. These results suggest that responses to the Rho-kinase inhibitors and cavernosal nerve stimulation are additive and are consistent with studies in which Y-27632 was injected intracavernosally after L -NAME was used to inhibit NOS in the rat (98). The present data are not in agreement with experiments in which the Rho-kinase inhibitor H-1152 was injected intraperitoneally and the response to cavernosal nerve stimulation was enhanced (159). The reason for the difference is uncertain

but may involve differences in experimental design, route of administration, or the selectivity of the Rho-kinase inhibitor used in the study.

To summarize, the results of the present study show that azaindole-1, a selective Rho-kinase inhibitor, had potent and long-lasting erectile activity in the anesthetized rat. Erectile responses to azaindole-1 were independent of: 1) NO released from NANC nerves innervating penile tissue, 2) NO released from muscarinic receptor stimulation and 3) sGC activation in the corpora cavernosa. These data show that the erectile response to azaindole-1 is not impaired after acute cavernosal nerve crush injury, however, these data also provided evidence that Rho-kinase inhibition is additive and does not potentiate NO-mediated erectile responses. These findings suggest that azaindole-1 may be useful in the treatment of ED when nonadrenergic, noncholinergic or cholinergic innervation is impaired, the NO-sGC-cGMP signaling pathway is inhibited, or cavernosal nerve injury has occurred.

IV.   Analysis of erectile responses to the sGC stimulator BAY 41-8543 under control and pathophysiological conditions

BAY 41-8543 is a heme-dependent, NO-independent stimulator of sGC. New findings in the present study are that BAY 41-8543 has significant erectile activity in vivo. BAY 41-8543 was less potent than sodium nitroprusside at

increasing ICP, however erectile responses to the sGC stimulator were longer in duration and had a greater AUC than responses to the NO donor. Erectile responses to BAY 41-8543 potentiated erectile responses to endogenously released and exogenously administered NO suggesting that this agent would be useful in the treatment of ED resulting from decreased NO bioavailability. The erectile response to cavernosal nerve stimulation was significantly reduced by muscarinic receptor antagonism with atropine, and the response to nerve stimulation was completely restored by intracavernosal administration of a low dose of BAY 41-8543.

Intracavernosal injections of the sGC stimulator BAY 41-8543 produced dose-related increases in ICP, ICP/MAP, AUC and response duration when injected into the corpora cavernosa in doses of 0.1–100 µg/kg. Increases in ICP were rapid in onset and long in duration (37 minutes) at the highest dose of the sGC stimulator studied. When erectile responses were compared on a nmol/kg basis, BAY 41-8543 was less potent than sodium nitroprusside and responses to the sGC stimulator were slower in onset and longer in duration than were responses to the NO donor.

The interaction between BAY 41-8543 and exogenous or endogenously released NO was investigated in vivo in the anesthetized rat. When administered together, BAY 41-8543 and the NO donor sodium nitroprusside produced a

significantly larger increase in ICP and AUC than the algebraic sum of the erectile responses to these agents when administered separately. These data show that BAY 41-8543 can synergize with exogenously administered NO in the corpora cavernosa of the anesthetized rat and suggest that low doses of the sGC stimulator and the NO donor could be used to enhance erectile activity. The interaction of BAY 41-8543 and endogenously released NO was also investigated and the increase in ICP and AUC in response to cavernosal nerve stimulation and intracavernosal injection of BAY 41-8543 was significantly greater than the algebraic sum of the erectile responses when the two erectile stimuli were administered separately.

Acute cavernosal nerve crush injury reduced the increase in ICP in response to cavernosal nerve stimulation by 83% and the response to cavernosal nerve stimulation was restored to pre-injury value by intracavernosal injection of a low dose of BAY 41-8543. These findings suggest that BAY 41-8543 may be useful in the treatment of ED resulting from iatrogenic nerve injury. These data indicate that BAY 41-8543 synergizes with endogenously released NO suggesting that BAY 41-8543 could be used to enhance erectile responses that are impaired in pathologic conditions where NO release or bioavailability from the nerves and/or endothelium is reduced (55, 170-172).

The results with BAY 41-8543 are consistent with studies using YC-1, the prototypical sGC stimulator. The intracavernosal injection of YC-1 increased ICP and intraperitoneal injections enhanced the response to cavernosal nerve stimulation in the rat (113). The results with BAY 41-8543 extend the results of previous studies with sGC stimulating agents by showing that intracavernosal injection of BAY 41-8543 can restore the response to cavernosal nerve stimulation after muscarinic receptor blockade and cavernosal nerve crush injury. These results suggest that treatment with BAY 41-8543 would be useful in situations where cholinergic nerve function is impaired.

In summary, intracavernosal injections of the sGC stimulator BAY 41-8543 increase ICP, ICP/MAP and AUC in a dose-related manner in the anesthetized rat. The erectile response to BAY 41-8543 was synergistic with endogenously released and exogenously administered NO. The increase in ICP in response to cavernosal nerve stimulation was attenuated by atropine and restored by intracavernosal injection of BAY 41-8543. BAY 41-8543 restored the erectile response to cavernosal nerve stimulation after cavernosal nerve crush injury. These data suggest that treatment with BAY 41-8543 would be useful in ED patients in which NO formation, release, or bioavailability are reduced by a disease process or when cholinergic or cavernosal nerve function are impaired by nerve injury.

V.   Analysis of erectile responses to the sGC activator BAY 60-2770 under
      control and pathophysiological conditions

Increased oxidative stress can decrease NO bioavailability in vascular

tissue through free radical interaction with vasoactive NO. Prolonged and

enhanced oxidative stress can alter the redox states of proteins that contain

electrophilic catalytic sites, such as the heme moiety of sGC, and reduce the

physiological function of these enzymes. Oxidation of the heme moiety on sGC

renders the enzyme incapable of inducing NO-mediated relaxation responses in

vascular smooth muscle. ODQ inhibits sGC by oxidizing the heme iron on the

enzyme and attenuates responses to NO and heme-dependent sGC stimulators

like BAY 41-8543 (132, 134). The catalytic activity of oxidized or heme-free sGC

can be restored with a new class of agents called sGC activators (Fig. 36) (59,

132, 173). Erectile responses to sGC activators have not yet been reported, and

new findings in the present study are that BAY 60-2770 has very potent erectile

activity in the anesthetized rat that is about 1,000-fold more potent than the sGC

stimulator BAY 41-8543 under control conditions. Interestingly, BAY 60-2770 has

very modest hypotensive activity in the systemic circulation and was more than 4

orders of magnitude less potent in its ability to decrease MAP compared with its

ability to increase ICP. These findings suggest that the activator has selectivity

for sGC in cavernosal and vascular smooth muscle within the penis when

compared to vascular smooth muscle of the systemic circulation (153).
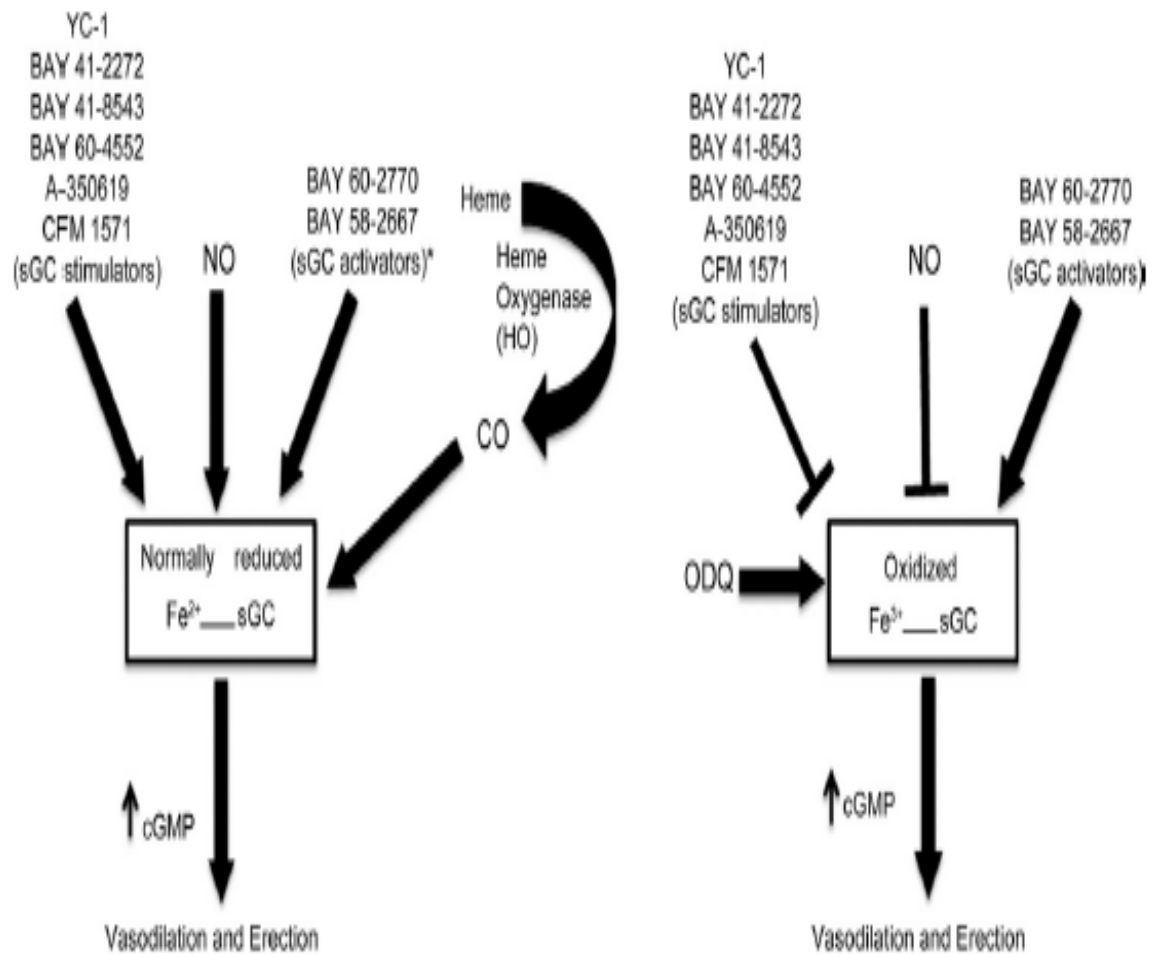
Figure 35. Effect of nitric oxide, carbon monoxide, soluble guanylate cyclase stimulators and soluble guanylate cyclase activators on reduced and oxidized soluble guanylate cyclase. Adapted from Lasker *et al.*, Am J Physiol Heart Circ Physiol 2013 (153).

In addition to having potent erectile activity in the rat under control conditions, the present data show that erectile responses to BAY 60-2770 were enhanced significantly by treatment with ODQ which attenuated erectile responses to two NO donor drugs and the sGC stimulator BAY 41-8543. The observation that increases in ICP, ICP/MAP, and AUC in response to BAY 60-2770 were enhanced by ODQ suggests that sGC activators would be effective in conditions where reactive oxygen species production is increased, NO is inactivated, and sGC is not responsive to NO or sGC stimulators.

The effect of NOS inhibition with L -NAME was investigated, and treatment with L -NAME in a dose that inhibited the response to cavernosal nerve stimulation by more than 90% did not attenuate the erectile response to BAY 60-2770. Taken together, the results with ODQ and L -NAME treatment suggest that BAY 60-2770 would have efficacy for treatment of ED in pathological conditions where NO production and bioavailability are diminished or when sGC is inactivated by oxidative stress. The ability of BAY 60-2770 to increase ICP, ICP/MAP, and AUC was not altered by acute or chronic nerve injury that reduced the response to cavernosal nerve stimulation by more than 80%. The results with acute and chronic nerve crush injury suggest that responses to BAY 60-2770 are not dependent on tonic cavernosal neurotransmission and that agents in this class may be useful in the treatment of ED when other treatments are not sufficient.

The final set of experiments in these studies provides evidence that sGC activators may be combined with existing therapies, namely PDE-5 inhibitors, for the treatment of ED in patients that are non-responsive to PDE-5 inhibitor therapy alone. Avanafil is a new, faster acting, orally bioavailable PDE-5 inhibitor that was approved by the FDA for the treatment of ED in 2012 (93). This agent has greater selectivity for the PDE-5 enzyme and therefore may have less side effects associated with inhibition of other phosphodiesterases. Avanafil pre-treatment significantly prolonged the erectile response to intracavernosal administration of the NO donor sodium nitroprusside (positive control). Pre-treatment with the PDE-5 inhibitor significantly prolonged the erectile response to the sGC activator BAY 60-2770. It was shown that the erectile duration to intracavernosal injection of BAY 60-2770 was potentiated to an even greater extent when the animal was pretreated with ODQ, providing evidence of substantially increased activity of the activator on oxidized sGC. These data suggest that sGC activators may be used alone and in combination with a PDE-5 inhibitor to improve erectile function in patients who do not respond to other forms of therapy.

In summary and as illustrated in figure 35, sGC activators increase the catalytic activity of normally reduced or oxidized sGC in vascular smooth muscle (110, 126, 132). In the present study the sGC activator BAY 60-2770 had very potent erectile activity compared with the sGC stimulator BAY 41-8543 and very modest hypotensive activity in the rat when injected intracavernosally (1-300

ng/kg), suggesting that the agent has selectivity for smooth muscle sGC in penile tissues. Erectile responses to the sGC activator BAY 60-2770 were significantly enhanced by treatment with ODQ, an agent that inhibits NO-stimulated sGC activity through oxidation of the heme iron on the enzyme. ODQ significantly reduced erectile responses to the NO donors sodium nitroprusside and DEA/NO and the sGC stimulator BAY 41-8543 by oxidizing the heme on sGC as reported by Zhao *et al.* (134). The potent erectile activity of BAY 60-2770 was not attenuated by treatment with the NOS inhibitor L -NAME or cavernosal nerve crush injury. Erectile responses to BAY 60-2770 under normal and oxidizing conditions were enhanced significantly with PDE-5 inhibitor pretreatment. These results suggest that agents such as BAY 60-2770, which increase the catalytic activity of oxidized or heme-free sGC, would potentially be useful in the treatment of pathological conditions where NO is inactivated by reactive oxygen species and sGC is oxidized with prolonged oxidative stress and not responsive to endogenously released or exogenous NO or sGC stimulators.

CONCLUSIONS

The results of the studies in this thesis show that:

1. Muscarinic receptor activation plays a significant role in the erectile
    response to cavernosal nerve stimulation in the anesthetized rat.
    Moreover, these results provide evidence that the dose of atropine used to
    block cholinergic signaling in the corpora cavernosa was selective for
    muscarinic receptors because erectile and vasodilator responses to the
    NO donor sodium nitroprusside were unaffected at a time when erectile
    and vasodilator responses to ACh were nearly abolished.

2. Erectile responses to intracavernosal injections of Rho-kinase inhibitors
    are not inhibited or prevented by antagonism of muscarinic receptors,
    inhibition of soluble guanylate cyclase or cavernosal nerve injury in the rat.
    These results provide evidence that pharmacological inhibition of Rho-
    kinase is additive and does not potentiate NO-mediated erectile responses
    in pathophysiological conditions of decreased NO bioavailability.

3.   Intracavernosal treatment with ODQ attenuates erectile responses to NO donor drugs and sGC stimulators. Our data suggest that ODQ treatment is selective because erectile responses to non-sGC-mediated vasodilators and the oxidation state of hemoglobin were not affected by use of this agent.

4.   The sGC stimulator BAY 41-8543 has significant erectile activity and can potentiate erectile responses to exogenous and endogenously released NO. These data shows that when cavernosal nerve-mediated erectile responses are attenuated with antagonism of muscarinic receptors or nerve crush injury, BAY 41-8543 can restore the responses to control value. These results suggest that BAY 41-8543 would be useful in the treatment of ED occurring from nerve damage post-prostatectomy.

5.   The sGC activator BAY 60-2770 has very potent erectile activity that is enhanced significantly in conditions of oxidative stress when erectile responses to endogenous NO or sGC stimulators are severely diminished. Additionally, duration of erectile responses to sGC activators may be enhanced further with concomitant PDE-5 inhibitor therapy providing

evidence that sGC activators can be used alone and in combination with existing treatments to improve erectile function in patients who are refractory to standard therapeutic options for ED.

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BIOGRAPHY

George Franklin Lasker was born on the 20th day of May 1982 in Atlanta, Georgia. Following graduation from high school in 2000, he worked full-time for a home improvement company. He began to take classes at a local community college in the evenings after work in 2004 and transferred to the Georgia Institute of Technology in Atlanta, Georgia, receiving his Bachelors of Science in Applied Biology in May of 2008. Additionally, he completed an honors thesis in molecular genetics and received the John H. Ridley Award for achievement in academics and scientific research as an undergraduate student. He moved to New Orleans, Louisiana and completed a Masters of Science in Pharmacology at the Tulane University School of Medicine in May of 2009. He was awarded the Physician Scientist Program Scholarship in March of the same year to attend Tulane and graduated from the School of Medicine with his M.D. and Ph.D. in May of 2016. He will begin his Neurological Surgery Residency at the University of California, San Francisco in June of 2016. He is the son of James and Linda Lasker of Marietta, Georgia and the older brother of Brandon, Jacquelyn and Julia Lasker.