Effects of 5-Cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine (BAY 41-2272) on Smooth Muscle Tone, Soluble Guanylyl Cyclase Activity, and NADPH Oxidase Activity/Expression in Corpus Cavernosum from Wild-Type, Neuronal, and Endothelial Nitric-Oxide Synthase Null Mice

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ABSTRACT

We aimed to characterize the relaxation induced by the soluble guanylyl cyclase (sGC) stimulator 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine (BAY 41-2272) and its pharmacological interactions with nitric oxide (NO) in the corpus cavernosum (CC) from wild-type (WT), endothelial nitric-oxide synthase (eNOS)/−/−, and neuronal (n)NOS/−/− mice. The effect of BAY 41-2272 on superoxide formation and NADPH oxidase expression was also investigated. Tissues were mounted in myographs for isometric force recording. Enzyme immunoassay kits were used for cGMP determination. sGC activity was determined in the supernatant fractions of the cavernosal samples by the conversion of GTP to cGMP. Superoxide activity was determined in the supernatant fractions of the cavernosal samples by the conversion of NADPH to superoxide.

Penile erection is the end result of corpus cavernosum (CC) smooth muscle relaxation initiated by nitric oxide (NO). This process is mediated in part by nitricergic nerves expressing neuronal NO synthase (nNOS), because sexual stimulation results in the peripheral release of NO. The neurally derived NO induces the initial dilation of the helicine resistance arterioles as well as the trabecular smooth muscle, resulting in an increased arterial blood inflow that further activates the endothelial NOS (eNOS), releasing an additional amount of NO down into the smooth muscle layers. Through the activation of soluble guanylyl cyclase (sGC), NO induces the enzymatic conversion of GTP to cGMP. In turn, cGMP acts as...
a modifying second messenger on ion channels, phosphodiesterases (PDE), and protein kinases, resulting in an erectile response (Andersson, 2001; Dean and Lue, 2005; Toda et al., 2005; Priviero et al., 2007).

Major risk factors for vascular diseases, including hypercholesterolemia (Shukla et al., 2005), atherosclerosis (Khan et al., 1999), and diabetes mellitus (Cartledge et al., 2000; Nangle et al., 2004), can interfere with the intricate vascular mechanisms underlying normal erection, and they are often associated with increased superoxide anion (O$_2^-$) formation in the vasculature. Superoxide reacts with NO to form peroxynitrite and other reactive nitrogen species, resulting in a diminished NO bioavailability (Jeremy et al., 2002; Förstermann and Münzel, 2006). Thus, the impaired NO-dependent cavernosal smooth muscle relaxation affects the normal erectile response, leading to erectile dysfunction. The excess production of O$_2^-$ is caused by the overexpression of endogenous vascular NADPH oxidase, and the up-regulation of this enzyme may play a role in the development of erectile dysfunction (Bischoff et al., 2003) as well as to cause human and animal erectile responses (Brioni et al., 2002; Mizusawa et al., 2002). In animal models, the NO-sGC-cGMP signaling is based on both insufficient NO-sGC-cGMP signaling, often associated with the use of conventional NO donors and the medical need to treat conditions associated with oxidative stress. The bensylindaozole derivate YC-1 was first reported to increase intracellular cGMP content in platelets (Ko et al., 1994), and it was later shown to stimulate soluble guanylate cyclase independently of nitric oxide to cause erectile responses (Brioni et al., 2002; Mizusawa et al., 2002). In animal models, the sGC stimulator BAY 41-2272 (Stasch et al., 2001) has been confirmed to be more potent than YC-1 and to elicit erectile responses in a conscious rabbit model of penile erection (Bischoff et al., 2003) as well as to cause human and rabbit corpus cavernous relaxation (Baracat et al., 2003; Kalsi et al., 2003).

In this investigation, we examined the in vitro effects of BAY 41-2272 in corpus cavernosum from mice with targeted deletions of NOS isoforms (nNOS$^{-/-}$ or eNOS$^{-/-}$), to understand the contribution of each isoform to the proerectile action of BAY 41-2272. First, we studied the effects resulting from modulation of the NO-signaling cascade on responses evoked by BAY 41-2272 as well as the sensitizing effects of this compound on both endogenous and exogenous NO-mediated cavernosal relaxation. Second, we investigated sGC expression and BAY 41-2272-induced enzyme activity in cavernosal tissue from these animals. Third, we studied the effects of BAY 41-2272 on both O$_2^-$ formation and NADPH oxidase expression in cavernosal tissue treated with the thromboxane A$_2$ analog U46619.

Materials and Methods

Animals. All experimental procedures were conducted in accordance with institutional guidelines, and they were approved by the Medical College of Georgia Institutional Animal Care and Use Committee. Male C57BL/6 mice (wild-type (WT) strain) and homozygous mutant mice lacking the gene for endothelial NOS (eNOS$^{-/-}$) or neuronal NOS (nNOS$^{-/-}$), 10 to 12 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in temperature-controlled facilities on a 12-h light/dark cycle with ad libitum food and water access.

Corpus Cavernosum Preparation. The animals were stunned by inhalation of CO$_2$, sacrificed by decapitation, and exsanguinated. The penises were surgically removed and placed in chilled Krebs-Henseleit buffer of the following composition: 130 mM NaCl, 14.9 mM NaHCO$_3$, 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH$_2$PO$_4$, 1.17 mM MgSO$_4$·7H$_2$O, and 1.6 mM CaCl$_2$·2H$_2$O. After removal of the glans penis and urethra, the penile tissue was cleaned from connective and adventitial tissues, and the fibrous septum separating the corpora cavernosa was opened from its proximal extremity toward the penile shaft. A slit was made in the tunica albuginea along the shaft to obtain two strips (11 x 1 x 1 mm) of CC from each animal. Each strip was mounted in a myograph for isometric force recording (Danish Myograph Technology, Aarhus, Denmark) coupled to a PowerLab 8/SP data acquisition system (software Chart 5.0; ADInstruments, Colorado Springs, CO). The bathing solution was maintained at 37°C, and it was continuously aerated with 95% O$_2$ and 5% CO$_2$. Tissues were allowed to equilibrate for 45 min under a resting tension of 2 mN. Repetitive supramaximal transmural electrical field stimulation (EFS) of autonomic nerves was delivered via platinum pin electrodes placed on either side of the cavernosal strips. Electrodes were attached to a stimulus splitter unit (Stimu-Splitter II; Med-Lab Instruments, Loveland, CO), which was connected to a Grass S88 stimulator (Astro-Med, West Warwick, RI).

Experimental Protocols. After equilibration, the ability of the preparations to develop contraction was assessed in 80 mM K$^+$-substituted Krebs-Henseleit solution. Next, endothelial function was assessed by applying 1 μM acetylcholine (ACh) in strips contracted with 10 μM phenylephrine (PE). In the first series of experiments, cumulative concentration-response curves to BAY 41-2272 (0.01–10 μM) were obtained in PE-precontracted CC in the absence or in the presence of 100 μM N$^\text{-}$nitro-$\text{-}$arginine methyl ester (l-NAME), 10 μM ODQ, or 0.1 mM sildenafil. One concentration-response curve was obtained in each CC, due to the lower contractile effect of PE observed in the second curve. Hence, control cavernosal preparations were run in parallel with experimental tissues. The pharmacological agents and the appropriate vehicles were added before PE exposure.

In the second series of experiments, relaxant responses to 0.01 to 10 μM ACh, 0.01 to 10 μM sodium nitroprusside (SNP), or acidified 10 to 300 μM NaN$_2$O solution (NO) were obtained in the absence and in the presence of increasing concentrations of BAY 41-2272 (0.01–0.1 μM). In the third series of experiments, relaxant responses to EFS (1–16 Hz; 20 V; 10-s trains) were obtained in tissues pretreated with bretylium tosylate and atropine to negate adrenergic and cholinergic stimulation, respectively. Next, the effects of increasing concentrations of BAY 41-2272 (as indicated above) were investigated on both the magnitude and duration of nitricergic-mediated relaxations.

Determination of Cavernosal cGMP Levels. Cavernosal tissues were equilibrated for 20 min in warmed and oxygenated Krebs’ solution, and then they were stimulated for 10 min with 0.01 to 1 μM BAY 41-2272, 1 μM SNP, or their combination. Preparations were collected immediately by freezing the segments in liquid nitrogen.
Cyclic GMP was extracted, and then it was quantified using commercially available kits (cyclic GMP enzyme immunoassay kit; Cayman Chemicals, Ann Arbor, MI) as described previously (Teixeira et al., 2005).

**Soluble Guanylyl Cyclase Activity.** Soluble guanylyl cyclase activity was determined in the precontracted supernatant fractions of the tissue samples by the conversion of GTP to cGMP. In brief, 30 μg of each protein sample were incubated for 10 min at 37°C in a total volume of 100 μl containing the following: 50 mM Tris-HCl, pH 7.4, 1 mM 3-isobutyl-1-methylxantine, 3 mM MgCl2, 0.5 mM GTP, 3 mM dithiothreitol, 5 mM phosphocreatine, and 0.25 mg/ml creatine kinase. The stimulation of enzyme activity was measured in the presence of 0.001 to 1 μM BAY 41-2272. The reaction was terminated by inactivation of sGC at 95°C for 10 min. Assays were also run in the presence of ODQ or SNP to inhibit or stimulate enzyme activity, respectively, to ensure the specificity of the reaction. cGMP was measured by using a commercially available cGMP enzyme-linked immunoassay according to the manufacturer’s instructions (Cyclic GMP ELISA kit; Cayman Chemicals).

**Effects of BAY 41-2272 on Superoxide Formation.** To assess the effect of U46619 in the absence or in the presence of BAY 41-2272 on O2•− formation, cavernosal segments were incubated with the thromboxane A2 analog U46619 for 1 or 8 h at 37°C in a 95% air, 5% CO2 incubator (Heraeus, Hera Cell; Kendro Laboratory Products, Langenselbold, Germany). Tissues were then equilibrated in Dulbecco’s modified Eagle’s medium with no phenol red for 10 min at 37°C in the incubator. Next, 20 mM horseradish cytochrome c (Sigma-Aldrich, St. Louis, MO), with or without 500 U/ml copper-zinc superoxide dismutase, was added, and the sample was incubated at 37°C in the incubator for 1 h. The reaction medium was removed, and reduction of the amount of cytochrome c was determined at 550 nm in an Anthos Lucy 1 spectrometer (Labotary-tech International, Ringmer, East Sussex, UK) and converted to millimoles of O2•− using a ΔE550 nm of 21.1 mmol-1•cm-1 as the extinction coefficient. The reduction of cytochrome c that was inhibitable with superoxide dismutase reflected actual O2•− release. Tissues were rinsed in phosphate-buffered saline, lysed with 0.1% (v/v) Triton X-100, and total protein content was measured using BCA Protein Assay kit (Pierce Chemical, Rockford, IL). The results were expressed as millimoles of O2•− per milligram of protein per hour. To determine whether the source of O2•− was NADPH oxidase, cavernosal tissues were incubated with the NADPH oxidase inhibitors diphenyleneiodonium chloride (DPI) and apocynin in parallel experiments. Furthermore, to determine the role of cGMP in mediating BAY 41-2272 effects, preparations were also incubated with the soluble guanylyl cyclase inhibitor ODQ.

**Semiquantitative RT-PCR.** Cavernosal tissue was cleared of connective tissue, and it was quickly snap-frozen in liquid nitrogen. Total RNA was isolated from each preparation with TRIzol reagent (Invitrogen, Carlsbad, CA). The amount of RNA was determined spectrophotometrically at A260. An amount of 0.5 μg of total RNA was reverse-transcribed [oligo(dT)12-18 primer using Moloney murine leukemia virus reverse transcriptase; GE Healthcare, Little Chalfont, Buckinghamshire, UK], and cDNAs were subsequently amplified in Ready-to-Go RT-PCR beads in a two-step procedure (GE Healthcare). Primer sequences (Connelly et al., 2003) for the sGCβ1 gene (GenBank accession no. AF297082) were as follows: forward, 5′-GCTCACCATTTGGAGCAGAGG-3′ and reverse, 5′-CCAGCTCTC-CACACTGCTG-3′. The PCR product was generated a fragment of 415 bp. Primer sequences for the sGCβ1 gene (GenBank accession no. AF020339) were as follows: forward, 5′-GACTGTCATCG-GAGAAGGG-3′ and reverse, 5′-CCAGGCATCGCTCTGTC-3′. The PCR product was generated a fragment of 309 bp. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. AF106860) were as follows: forward, 5′-GCTGTCCTCT CCTGTCGACA-3′ and reverse, 5′-GCCTCAGAGGATGTTGAT-3′. The PCR product generated a fragment of 199 bp, which was used as an internal control. The general protocol for PCR amplification was followed in 35 cycles at 94°C for 1 min, at 60°C for 1 min, and 72°C for 1 min followed by a final extension at 72°C for 10 min. Amplified DNA fragments were separated in a 2% agarose gel, and then they were stained with ethidium bromide. The gel images were recorded by video camera (Sony CCD Video Module; Sony, Tokyo, Japan) connected to an IBM AT computer (IBM, New York, NY) with a 512 × 512 pixel array imaging board with 256 gray levels. The PCR products were quantified by densitometric scanning of gel images using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). Results were then expressed as the densitometric ratio of gene of interest/CAPDH.

**Western Blot Analysis.** The muscle strips were homogenized in a lysing buffer containing 40 mM HEPES, 1% Triton X-100, 10% glycerol, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride. The tissue lysate was centrifuged at 10,000 g, and the supernatant was collected. The protein concentration was determined using a SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins were subsequently transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked by treatment with 5% milk in Tris-buffered saline containing 0.05% Tween 20, and then they were probed with antibodies against sGCα1 (1:100), sGCβ1 (1:200), gp91phox (1:500), p47phox (1:500), p22phox (1:500), or p67phox (1:500) and incubated with a horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence autoradiography. The bands were quantified by densitometric scanning of film images using UN-SCAN-IT software (Silk Scientific Inc.). Results are expressed as the densitometric ratio of protein of interest/β-actin (1:2000).

**Drugs and Chemicals.** Acetylsalicylic, apocynin, ω-conotoxin GVIA, cromakalim, U46619, diethylamine NONOate, DPI, L-NAME, ODQ, phenylephrine, sodium nitrite (NaNO2), SNP, and tetrodotoxin was purchased from Sigma-Aldrich. Sildenafil citrate was obtained from Pfizer (New York, NY). BAY 41-2272 was obtained from Axon Life Sciences, Inc. (San Diego, CA). The antibodies used to probe for sGCα1 and sGCβ1 were purchased from Millipore Corporation (Billerica, MA). The antibodies used to probe for gp91phox, p22phox, p47phox, and p67phox were obtained from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA). The antibody used to probe for β-actin was acquired from Sigma-Aldrich. All other reagents were of analytical grade.

**Statistical Analysis.** All values of relaxation are shown as a percentage of the level of precontraction. All data are expressed as means ± S.E.M. (n). Statistical comparisons were made using two-way analysis of variance and Student’s t test with p < 0.05 taken as significant in each case. EC50 values are presented as the negative logarithm (pEC50) and they were calculated by fitting concentration-response relationships to a sigmoidal model of the form log-concentrations versus response using GraphPad software, version 3.00 (GraphPad Software Inc., San Diego, CA).

**Results**

**Relaxant Responses Induced by BAY 41-2272.** The contractions induced by high K+ solution were not significantly different in corpus cavernosum from either strain (WT, 2.0 ± 0.5 mN; eNOS−/−, 2.3 ± 0.5 mN; nNOS−/−, 1.9 ± 0.4 mN). Isolated cavernosal segments were contracted with 10 μM PE, to achieve a level of tension representing 70 to 80% of the reference contraction to 80 mM KCl. There was no significant difference in the level of contraction at the chosen PE concentration (data not shown). The cumulative addition of 0.01 to 10 μM BAY 41-2272 caused similar concentration-dependent and long-lasting relaxations in PE-contracted tissues from WT and nNOS−/− mice, with pEC50 values of 6.36 ± 0.07 and 6.27 ± 0.06, respectively (n = 16). When
applied to cavernosal strips from eNOS−/− animals, the relaxations to BAY 41-2272 were significantly less potent, with a pEC50 value of 5.88 ± 0.07 (p < 0.01; Fig. 1).

Effects of ODQ, l-NAME, and Sildenafil on BAY 41-2272-Evoked Relaxations. Inhibition of sGC with 10 μM ODQ (Fig. 2; n = 6) resulted in a significant reduction of relaxations elicited by BAY 41-2272, as evidenced by the significant rightward shifts 5.4-, 4.2-, and 2.5-fold in cavernosal strips from WT (p < 0.01), nNOS−/− (p < 0.01), and eNOS−/− (p < 0.01) mice, respectively. In the concentration used in this study, ODQ virtually abolished maximal relaxations elicited by 10 μM ACh (control (CTL), 78 ± 4%; ODQ, 1 ± 1%; p < 0.01; n = 5), 10 μM SNP (CTL, 90 ± 3%; ODQ, 5 ± 2%; p < 0.01; n = 6), and 300 μM NO (CTL, 87 ± 5%; ODQ, 13 ± 5%; p < 0.01; n = 6) in preparations from WT mice.

The relaxant responses mediated by BAY 41-2272 were significantly inhibited in 100 μM l-NAME (Fig. 2; n = 5)-treated cavernosal strips from WT and nNOS−/− mice (2.8- and 3-fold rightward shifts, respectively), without any appreciable effects on maximal responses. Nonetheless, l-NAME failed to cause similar decreases in the sensitivity to BAY 41-2272 in tissues from eNOS−/− mice. In addition, l-NAME fully blocked relaxations induced by 10 μM ACh (CTL, 70 ± 3%; l-NAME, 3 ± 3%; p < 0.01; n = 5), without affecting those elicited by 10 μM SNP (CTL, 94 ± 2%; l-NAME, 96 ± 5%; n = 5).

Application of the selective PDE5 inhibitor sildenafil at 0.1 μM (n = 5) to the bathing medium significantly potentiated (p < 0.01) the relaxations induced by BAY 41-2272 in WT (CTL, 6.35 ± 0.07; sildenafil, 6.90 ± 0.06), nNOS−/− (CTL, 6.28 ± 0.07; sildenafil, 6.78 ± 0.04), and eNOS−/− (CTL, 5.72 ± 0.08; sildenafil, 6.47 ± 0.07) animals. Sildenafil also enhanced the relaxant responses induced by SNP and NO in all groups (p < 0.01; n = 5 each). The relaxations induced by BAY 41-2272 were not affected by blockade of Na+ or N-type Ca2+ channels, because 1 μM tetrodotoxin and 1 μM ω-conotoxin GVIA, respectively, failed to affect these responses (data not shown).

Enhancement of ACh and SNP Relaxations by BAY 41-2272. Increasing concentrations of BAY 41-2272 (0.01–0.1 μM) caused significant enhancement of 0.01 to 10 μM ACh-induced relaxations. At the concentrations of 0.03 and 0.1 μM, BAY 41-2272 significantly augmented the magnitude of 0.01 μM (CTL, 12 ± 1%; BAY 41-2272, 19 ± 2 and 24 ± 3% at 0.03 and 0.1 μM, respectively) and 0.1 μM (CTL, 49 ± 3%; BAY 41-2272, 62 ± 3 and 68 ± 4% at 0.03 and 0.1 μM, respectively). ACh responses in CC from WT mice (p < 0.01; n = 6). Similar results were obtained in CC from nNOS−/− animals (n = 5). ACh failed to evoke relaxations in CC from eNOS−/− mice, as expected. The relaxant responses of CC induced by SNP in WT animals were shifted to the left as a function of increasing concentrations of BAY 41-2272 (n = 5), by approximately 1.3-, 2.1-, and 3.2-fold for 0.01 μM (CTL, 6.57 ± 0.05; BAY 41-2272, 6.70 ± 0.07), 0.03 μM (CTL, 6.57 ± 0.05; BAY 41-2272, 6.89 ± 0.07; p < 0.05), and 0.1 μM (CTL, 6.57 ± 0.05; BAY 41-2272, 7.08 ± 0.05; p < 0.01) of the compound, respectively. In cavernosal segments from both nNOS−/− and eNOS−/− mice, BAY 41-2272 caused similar leftward shifts in the concentration-response curves elicited by SNP.

Enhancement of EFS and NO Relaxations by BAY 41-2272. In cavernosal strips from WT and eNOS−/− pre-treated with 30 μM bretylium tosylate and 1 μM atropine to prevent the effects of adrenergic and cholinergic transmission, EFS (1–16 Hz) elicited frequency-related relaxations that were abolished by either 1 μM tetrodotoxin (n = 5), 100 μM l-NAME (n = 5), or 10 μM ODQ (n = 5), confirming the nitrergic nature of these responses. EFS responses were also largely reduced following treatment with 1 μM ω-conotoxin GVIA (n = 4). Cavernosal segments from nNOS−/− mice failed to relax in response to EFS, as expected. As shown in Fig. 3, 0.1 μM BAY 41-2272 (n = 6) enhanced the magnitude of relaxation evoked by lower frequencies of stimulation (1–4 Hz; p < 0.05) in CC from WT animals (p < 0.01). Most importantly, the duration of the nitrergic responses (time elapsed from 50% relaxation to 50% recovery) was markedly potentiated by treatment with 0.03 to 0.1 μM BAY 41-2272 over the full range of the frequency-response curve. Similar results were obtained in tissues from eNOS−/−. Addition of noncumulative concentrations of NO (as acidified NaNO3), caused transient relaxations, whose magnitude and duration

Fig. 1. Representative trace (a) of a concentration-response curve to 0.01 to 10 μM BAY 41-2272 in corpus cavernosum from WT mice contracted with 10 μM PE. Concentration-response curves to BAY 41-2272 (n = 16) in corpus cavernosum strips from WT (closed circles), nNOS−/− (open circles), and eNOS−/− (closed squares) mice contracted by PE. Experimental values were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. The corresponding pEC50 values are represented in the panel. Data represent the mean ± S.E.M. of n experiments. **, p < 0.01 compared with curves elicited in WT and nNOS−/− strips.
were both enhanced following treatment with 0.03 to 0.1 μM BAY 41-2272 (n = 4). Furthermore, BAY 41-2272 at the same concentrations failed to enhance the corpus cavernous relaxation evoked by the K⁺ channel opener cromakalim (pEC₅₀ values of 6.27 ± 0.07 in the absence and 6.39 ± 0.06 in the presence of 0.1 μM BAY 41-2272; n = 4).

Effect of BAY 41-2272 on Cavernosal cGMP Levels. Table 1 shows that the basal cGMP content (picomoles per milligram of protein) of eNOS⁻/⁻ strips (0.04 ± 0.03) was significantly lower (p < 0.05) than WT (0.14 ± 0.04) or nNOS⁻/⁻ (0.17 ± 0.03) tissues (n = 4). In strips treated with 1 μM SNP or 0.01 to 1 μM BAY 41-2272, the cGMP levels were significantly increased above control values (p < 0.01). In tissues treated with a combination of SNP and BAY 41-2272, the resulting intracellular cGMP levels were markedly above the sum of their effects alone (p < 0.01). Treatment with 10 μM ODQ caused marked reductions in BAY 41-2272-stimulated increases in cGMP levels. Incubation of strips from WT or nNOS⁻/⁻ mice with 100 μM L-NAME resulted in a basal cGMP concentration similar to that found in tissues from eNOS⁻/⁻ animals (0.04 ± 0.01 and 0.05 ± 0.03 pmol/mg protein, respectively).

Effect of BAY 41-2272 on Soluble Guanylyl Cyclase Activity. To confirm that the functional responses elicited by the BAY 41-2272 correlated with enzyme stimulation, we examined the effects of this compound on cGMP levels in supernatant crude fractions of mouse cavernosus smooth muscle, using 0.5 mM GTP as substrate to measure sGC activity. Basal enzyme activity and maximal increase in cGMP formation by BAY 41-2272 did not differ among WT, nNOS⁻/⁻, and eNOS⁻/⁻ samples (Fig. 4; n = 3). In addition, the sensitivity of sGC to the NO donor diethylamine NONOate was not different for half-maximal activation of the enzyme (pEC₅₀ values, WT, 6.51 ± 0.11; nNOS⁻/⁻, 6.32 ± 0.14; eNOS⁻/⁻, 6.39 ± 0.16; n = 3). In presence of 10 μM ODQ, BAY 41-2272-stimulated maximal increases in cGMP formation were reduced to 20 ± 7, 17 ± 8, and 22 ± 11% of values in the absence of the inhibitor in WT, nNOS⁻/⁻, and eNOS⁻/⁻ samples. In addition, ODQ failed to affect basal sGC activity in WT (CTL, 2.96 ± 0.12; ODQ, 2.81 ± 0.09 pmol cGMP/mg/min), nNOS⁻/⁻ (CTL, 3.03 ± 0.15; ODQ, 2.99 ± 0.14 pmol cGMP/mg/min), and eNOS⁻/⁻ (CTL, 2.61 ± 0.11; ODQ, 2.77 ± 0.19 pmol cGMP/mg/min) samples. In contrast, the coinubcation with the EC₅₀ concentration of diethylamine NONOate (0.3 μM) resulted in an approximate 100-fold stimulation of sGC, compared with baseline levels in all groups (p < 0.01; n = 3). In the absence of either the protein extract or GTP, no significant formation of cGMP was observed.

Soluble Guanylyl Cyclase Expression Studies. Complimentary DNAs prepared by reverse transcription of total RNA extracted from mouse cavernosae segments coding for sGCα1 and sGCβ1 subunits were detectable. Expression of sGC subunit mRNAs in cavernosal tissue from WT, eNOS⁻/⁻, and eNOS⁻/⁻ mice was similar when normalized to the GAPDH content of the samples. Figure 5 demonstrates the electrophoretic visualization of the PCR products obtained from the amplification of cDNAs. In all samples, the fragments exhibiting the expected sizes were amplified without any contamination from nonspecific products (415 bp for...
sGCα1 and 309 bp for sGCβ1; n = 6). Evaluation of cavernosal sGC protein expression was assessed by Western blot using antibodies directed against the α1 (77-kDa) and β1 (72-kDa) subunits of mouse sGC (Fig. 5). Quantification of 6 blots from six different pairs of cavernosal sGC preparations from WT, nNOS−/−, and eNOS−/− by densitometry resulted in a similar content of sGC subunits in these three groups, when normalized to the β-actin content of the samples.

### Inhibitory Effect of BAY 41-2272 on NADPH Oxidase Activity and Expression.

The thromboxane A2 analog U46619 at 0.01 M was a potent stimulator of superoxide formation by cavernosal tissues incubated for both 1 and 8 h.

### Table 1

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<th>Cyclic GMP levels represent the mean ± S.E.M. of n experiments performed in duplicate.</th>
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<td>pmol/mg protein</td>
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<td>Vehicle</td>
<td>0.14 ± 0.04</td>
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<td>BAY, 0.01 μM</td>
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<td>BAY, 0.1 μM</td>
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<td>1.44 ± 0.56*</td>
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<td>BAY, 1 μM + ODQ</td>
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*p < 0.05 and **p < 0.01 compared with the respective vehicles.

*p < 0.05 compared with the sum of BAY 41-2272 plus SNP values alone.

Fig. 3. Effects of increasing concentrations (0.01–0.1 μM; n = 6) of BAY 41-2272 on the magnitude and duration of nitricergic relaxations evoked by EFS (1–16 Hz) in mouse cavernosal strips from WT (a and b) and eNOS−/− (c and d) mice treated with 10 μM PE. Nitricergic duration was determined as the time elapsed from 50% relaxation to 50% recovery. Data represent the mean ± S.E.M. of n experiments. *p < 0.05 and **p < 0.01 compared with the respective CTL values.

E, representative tracing of the effects of 0.1 μM BAY 41-2272 on nitrergic relaxations of a cavernosal strip from WT animals (4 Hz; 30-s trains). The preparation was stimulated at 30-s intervals for 30 s. Muscle tone was raised with 10 μM PE in the absence and 50 μM PE in the presence of BAY 41-2272.
and protein, indicates that the prototypical \(a1/\beta1\) heterodimeric enzyme is expressed in the mouse corpus cavernosum. BAY 41-2272 stimulates sGC in a synergistic manner with NO, as indicated by the enhancement of endogenous (EFS and ACh) and exogenous (SNP and acidified NaNO\(_2\)) NO-induced relaxations, as well as by the increases in cGMP concentration. Most notably, the results indicate that BAY 41-2272 also inhibits \(O_2^*\) formation and NADPH oxidase expression in the corpus cavernosum, providing additional insight into the functional importance of this compound in the management of erectile dysfunction.

BAY 41-2272 produces erectogenic NO-dependent responses in a conscious rabbit model (Bischoff et al., 2003), and it causes cavernosal smooth muscle relaxation in penile tissue from both humans and rabbits (Baracat et al., 2003; Kalsi et al., 2003). Other studies demonstrate that BAY 41-2272 causes cGMP-dependent relaxant responses in a variety of vascular (Evgenov et al., 2004; Bawankule et al., 2005; Priviero et al., 2005; Teixeira et al., 2006b,c) and non-vascular smooth muscles (Teixeira et al., 2006a; Belik et al., 2007). Accordingly, consistent with its ability to directly stimulate sGC, BAY 41-2272 produced a complete relaxation of phenylephrine-precontracted corpus cavernosum preparations. The concentration-dependent relaxations were accompanied by increases in intracellular cGMP levels, corroborating the above-mentioned reports.

The relaxant activity of BAY 41-2272 in the corpus cavernosum has been shown to be dependent on NO, because blockade of its synthesis caused inhibition of BAY 41-2272 relaxations (Baracat et al., 2003; Kalsi et al., 2003). Several publications suggest that erection is mainly evoked by NO liberated from nitrergic nerves in the corpus cavernosum (Ignarro et al., 1990; Burnett et al., 1992; Hurt et al., 2006), although its release from the endothelium is postulated to be involved in the relaxation of the cavernosal smooth muscle (Hurt et al., 2002). To further elucidate the involvement and relative importance of neurally and endothelium-derived NO, BAY 41-2272-induced erectile responses were quantified in mice lacking eNOS (eNOS\(^{−/−}\)) or nNOS (nNOS\(^{−/−}\)) and in WT controls. Our results show that BAY 41-2272 is approx-
imately 3-fold less potent in causing relaxation in corpus cavernosum from eNOS−/− mice compared with WT and nNOS−/−, suggesting that NO released from the endothelium, but not from nerves, partially account for the relaxant effect of this drug in cavernosal tissue. A decreased sGC expression in eNOS−/− can be ruled out, because no differences in expression were observed in cavernosal tissue from WT and NOS null mice. The findings that L-NAME inhibits...
BAY 41-2272 relaxations in corpus cavernosum from WT and nNOS\(^{-/-}\), but not eNOS\(^{-/-}\), further support a role for endothelium-derived NO in these responses. cavernosal tissues from WT and nNOS\(^{-/-}\) treated with l-NAME present a marked reduction in basal intracellular cGMP levels, comparable with those obtained in eNOS\(^{-/-}\) corpus cavernosum, further indicating that NO is constantly released from the sinusoidal endothelial cells to regulate the trabecular smooth muscle tone. In addition, blockade of nitricergic transmission with tetrodotoxin (Na\(^+\) channel blocker) or α-conotoxin GVIA (N-type Ca\(^{2+}\) channel blocker) failed to reduce BAY 41-2272-evoked relaxations, ruling out a role for neurogenic NO in the relaxant activity of this drug.

One major prerequisite for the NO-induced activation of sGC is the presence of the reduced Fe\(^{2+}\) heme moiety, because its removal abolishes any NO-induced enzyme activation (Foerster et al., 1996). In addition to removal of the heme, its oxidation by sGC inhibitors such as ODQ leads to the formation of an NO-insensitive form of the enzyme (Schrammel et al., 1996). The cysteine 238 and cysteine 243 spanning region in the \(\alpha\) subunit of sGC was identified as part of the target site for BAY 41-2272, in addition to the heme group (Stasch et al., 2001). Our results clearly showed that BAY 41-2272-induced relaxations and increased cGMP levels occurred in an ODQ-sensitive manner, in agreement with previous studies wherein ODQ inhibited BAY 41-2272-induced stimulation of recombinant sGC (Stasch et al., 2001) and vasorelaxation (Priviero et al., 2005; Teixeira et al., 2006b,c). In contrast, inhibition of cGMP degradation with sildenafil significantly enhanced BAY 41-2272-evoked relaxations, further ensuring the role of cGMP in these relaxant responses, as observed in previous studies (Priviero et al., 2005; Teixeira et al., 2006a,b,c).

The finding that BAY 41-2272-induced relaxations of corpus cavernosum from eNOS\(^{-/-}\) mice were less sensitive to inhibition by ODQ (approximately 2-fold) also reflects that BAY 41-2272 and NO released from endothelial cells act in conjunction to evoke relaxation. Indeed, stimulation of sGC isolated from WT, nNOS\(^{-/-}\), and eNOS\(^{-/-}\) by BAY 41-2272 results in similar levels of enzyme activity, which were reduced to similar extents by addition of ODQ to the reaction mixture. In contrast, ODQ did not significantly affect basal enzyme activity, as expected (Friebe and Koesling, 2003). Another interesting finding was the evident discrepancy between the inhibitory effects of ODQ on relaxant responses and cGMP levels. Because BAY 41-2272 does not stimulate cAMP production in the smooth muscle (Teixeira et al., 2006a,b), cGMP-independent mechanism such as inhibition of Ca\(^{2+}\) entry or activation of protein phosphatases might explain this discrepancy (Teixeira et al., 2006b,c).

BAY 41-2272 can act as an allosteric activator in the absence of NO, leading to even more pronounced sGC activation in the presence of NO. Indeed, BAY 41-2272 and NO donors synergize over a wide range of concentrations to stimulate enzyme activity in assays using purified sGC (Stasch et al., 2001; Koglin et al., 2002). Likewise, we have successfully demonstrated a synergistic interaction between BAY 41-2272 and diethylamine NONOate to increase cGMP in sGC activity assays using protein supernatants obtained from corpus cavernosum from WT, nNOS\(^{-/-}\), and eNOS\(^{-/-}\) mice. Functionally, this synergistic interaction was represented by the potentiation of ACh- (releases NO from the endothelium), NO- (as acidified NaNO\(_2\) solution), and SNP (donates NO)-induced relaxations, further indicating that BAY 41-2272 and NO activate sGC by different mechanisms. Actually, BAY 41-2272 and SNP caused marked increases in intracellular cGMP levels, such as that their coincubation with the cavernosal tissue resulted in much higher cGMP production compared with their effects alone, similar to results obtained in rat mesenteric artery (Teixeira et al., 2006c), basilar artery (Teixeira et al., 2006b), and anococcygeus muscle (Teixeira et al., 2006a). BAY 41-2272 also consistently enhanced the magnitude and duration of EFS-induced nitricergic relaxations, supporting the concept that BAY 41-2272 synergizes with endogenous NO (Teixeira et al., 2006a). The effect on nitricergic duration was much more pronounced than that on the amplitude of the responses. Extrapolated to an in vivo situation, an increased duration would probably correspond to a prolonged corpus cavernosum relaxation, and hence erection.

NO is a potent inhibitor of NADPH oxidase expression, an effect mediated by a cGMP-dependent mechanism (Muzaffar et al., 2004b). To the best of our knowledge, the present study is the first to demonstrate that BAY 41-2272 is a potent inhibitor of \(O_2^-\) formation in mouse corpus cavernosum treated with the thromboxane A\(_2\) analog U46619, both acutely (1 h) and in the long term (8 h). Most importantly, BAY 41-2272 also negatively regulated the expression of p22\(^{phox}\) and gp91\(^{phox}\) (active catalytic subunits of NADPH oxidase) in the prolonged incubation. Because NADPH oxidase generates \(O_2^-\), which reacts with NO to form reactive nitrogen species, these data indicate that thromboxane A\(_2\) may cause erectile dysfunction by negating the bioavailability of NO. The inhibitory effect on both NADPH oxidase activity and expression was cGMP-dependent, because these activities of BAY 41-2272 were fully prevented by ODQ. Moreover, this inhibition was achieved within the range of concentrations required to elicit cavernosal relaxations, stimulate sGC activity, and increase intracellular cGMP levels, further ensuring that the inhibitory effect is mediated through stimulation of sGC. Thus, the inhibition of NADPH oxidase and the concomitant reduction in \(O_2^-\) formation by BAY 41-2272 also enhances the effects of endogenously produced NO. Therefore, this antioxidant effect of BAY 41-2272 may constitute an additional mechanism to counteract erectile dysfunction, particularly in diseases related to oxidant stress, such as diabetes, atherosclerosis, and hypercholesterolemia. In this context, it is reasonable to suggest that this antioxidant effect of BAY 41-2272 may augment the therapeutic effect of the drug.

In conclusion, the present study demonstrated that both expression and activity of sGC are not altered in corpus cavernosum from mice with targeted deletions of either nNOS or eNOS. BAY 41-2272 causes corpus cavernosum relaxation in a synergistic manner with endogenous or exogenous NO, with concomitant increases in intracellular cGMP levels. Our results also demonstrate that in the course of stimulation of sGC, BAY 41-2272 has a potent inhibitory effect on \(O_2^-\) formation through a reduction of both NADPH oxidase expression and activity. Taken together, such properties render BAY 41-2272 as a promising therapeutic drug to treat erectile dysfunction, particularly in the event of an increased intrapenile oxidative stress.
References

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