Beneficial Effect of the Soluble Guanylyl Cyclase Stimulator BAY 41-2272 on Impaired Penile Erection in \(\text{db/db}^{-/-}\) Type II Diabetic and Obese Mice

Kenia Pedrosa Nunes, Cleber E. Teixeira, Fernanda B. M. Priviero, Haroldo A. Toque, and R. Clinton Webb

Department of Cell and Regenerative Biology, University of Wisconsin, Madison, Wisconsin (K.P.N.); Laboratory of Multidisciplinary Research, Universidade São Francisco, Bragança Paulista, Brazil (F.B.M.P.); and Departments of Physiology (C.E.T., R.C.W.) and Pharmacology and Toxicology (H.A.T.), Georgia Regents University, Augusta, Georgia

Received October 22, 2014; accepted March 4, 2015

ABSTRACT

Type 2 diabetes mellitus (DM2) and obesity are major risk factors for erectile dysfunction (ED). In diabetes, increased oxidative stress leads to decreased nitric oxide (NO) bioavailability, and diabetic patients appear to be less responsive to conventional therapy with phosphodiesterase type 5 inhibitors. We investigated whether the soluble guanylyl cyclase stimulator BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4ylamine) is effective in improving impaired corpus cavernosum (CC) relaxation in obese DM2 mice by reducing oxidative stress. Adult \(\text{db/db}^{-/-}\) mice or their lean \(\text{db}/+\) littermates were used to assess vascular function, cGMP levels, antioxidant status, NADPH oxidase expression, and superoxide formation in the absence or presence of BAY 41-2272. Results showed that BAY 41-2272 (10\(^{-8}\) to 10\(^{-5}\) M) potently relaxed CC from \(\text{db}/+\) or \(\text{db/db}^{-/-}\) mice in a similar manner. BAY 41-2272 significantly enhanced both endothelium-dependent and nitricergic relaxation induced by electrical field stimulation (EFS), and improved the impaired relaxation to acetylcholine and EFS in the diabetic animals in a concentration-dependent manner (10\(^{-8}\) to 10\(^{-7}\) M). BAY 41-2272 increased cGMP levels and potentiated relaxation responses to exogenous NO in CC. Total antioxidant status was reduced in plasma and urine whereas expression of vascular NADPH oxidase subunits (gp91phox, p22phox, and p47phox) was increased in the CC of \(\text{db/db}^{-/-}\) mice, suggesting a state of oxidative stress. These effects were prevented by BAY 41-2272 in a concentration-dependent manner. These results suggest that BAY 41-2272 improves CC relaxation in \(\text{db/db}^{-/-}\) mice by increasing cGMP and augmenting antioxidant status, making this drug a potential novel candidate to treat ED.

Introduction

Diabetic men are three times more likely to suffer from erectile dysfunction (ED) than nondiabetic men (Moore and Wang, 2006). The risk of ED is even higher when diabetes is associated with obesity (Feeley and Traish, 2009). Diabetes and obesity are the most common risk factors for ED (Bacon et al., 2006; Giugliano et al., 2010) because of their deleterious effects on the vasculature (Tamlor, 2009). Increased reactive oxygen species (ROS) production and oxidative stress have also been documented in ED, contributing to penile vascular dysfunction (Silva et al., 2014). Many studies have shown that ED in type 2 diabetes mellitus (DM2) is associated with a hypercontractile state of the penile smooth muscle, which impairs endothelial function and leads to cavernosal veno-occlusive dysfunction (Chitaley, 2009; Hidalgo-Tamola and Chitaley, 2009). Also, increased ROS generation results in oxidative stress that contributes to vascular dysfunction via nitric oxide (NO) scavenging and other direct or indirect mechanisms, which have long been implicated in diabetic vascular complications.

A normal erection is a complex mechanism that requires a perfect balance between corpus cavernosum (CC) relaxation and contraction. NO is a key signaling molecule for relaxation of the cavernosal tissue and subsequently normal penile erection (Grazzki et al., 2010; Andersson, 2011). The gaseous NO molecule, with major vasodilatory effects, is derived from neuronal nitric oxide synthase (nNOS) and endothelial NOS. Once released from nitrergic nerve endings and the endothelium lining the arteries supplying the penis and the cavernosal sinusoid spaces, NO diffuses into the smooth muscle layers and activates the soluble guanylyl cyclase (sGC) in vascular...
smooth muscle cells. Activity of sGC promotes the enzymatic conversion of GTP to cGMP, which in turn induces smooth muscle relaxation via lowering intracellular calcium levels as well as activation of ion channels and protein kinases that further reduce the contractile state of the penis, thereby promoting and maintaining the erectile response (Nunes and Webb, 2012). In diabetes, endogenous NO, released from nitrergic nerves in the CC, and activation of the NO-sGC-cGMP pathway are significantly diminished (Chitaley, 2009; Hidalgo-Tamola and Chitaley, 2009; Angulo et al., 2010).

Phosphodiesterases (PDE) increase the degradation of cGMP. PDE type 5 (PDE-5) inhibitors, which produce an NO-dependent increase in intracellular cGMP concentration, are the primary therapeutic approach for ED. However, more than 30% of diabetic patients with ED do not respond to PDE-5 inhibitor therapy. For these patients, endogenous NO production may be so impaired that inhibition of cGMP degradation does not provide a significant benefit (McMahon et al., 2006). Currently, two different classes of drugs have been developed that directly target sGC, increasing cGMP formation and promoting penile erection. These agents are called sGC stimulators and sGC activators (Becker et al., 2001; Lasker et al., 2010, 2013). The sGC stimulators are agents that directly activate sGC and increase its catalytic activity independent of NO availability, thereby increasing cGMP formation and leading to penile erection (Eugenov et al., 2006).

BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine) is an sGC stimulator that has been shown to produce antiproliferative and vasodilatory effects (Eugenov et al., 2006), as well as to potentiate erectile responses (Bischoff et al., 2003) and relax the CC of humans and animals (Baracat et al., 2003; Kalsi et al., 2003; Claudino et al., 2011). This compound was suggested to have a high potency and no PDE inhibitory activity (Stasch et al., 2001). In a NO-deficient rat model, long-term oral treatment with BAY 41-2272 improved the impaired cavernosal relaxation (Claudino et al., 2011). In a previous investigation of the effects of BAY 41-2272 in mice CC, our group showed that this compound reverses the increased NADPH oxidase-dependent superoxide generation by decreasing protein expression of its subunits gp91phox and p22phox (Teixeira et al., 2007).

The NADPH oxidase enzyme complex is composed of a membrane-bound cytochrome, which includes subunits gp91phox and p22phox, and a cytosolic component composed of five subunits, including p47phox (Lassegue and Clempps, 2003). Upregulation of gp91phox contributes to ED in conditions where decreased NO availability is prolonged (Claudino et al., 2010). Oxidative stress, mediated through NADPH oxidase, plays a crucial part in the pathology of vascular diseases including ED (Jin et al., 2008). In diabetes, increased superoxide anion (O2·−) formation in the vasculature interferes with the complex mechanisms underlying normal penile erection. The excessive production of O2·− is mainly caused by overexpression of endogenous vascular NADPH oxidase which contributes to the development of ED (Burnett et al., 2006).

BAY 41-2272, but not a PDE-5 inhibitor, enhances the nitrergic relaxation response in anococcygeus and retractor penile muscle (Kalsi et al., 2004) (ideal tissues to study nitrergic neurotransmission), which are impaired in streptozotocin-induced diabetic rats (Cheah et al., 2002). These data suggest that endogenous NO from nitrergic nerves is decreased in diabetes and show that sGC stimulators are more effective than PDE-5 inhibitors in the treatment of diabetes-induced ED.

To the best of our knowledge, there are no previous studies investigating the action of BAY 41-2272 on diabetic CC. Additionally, few studies have been performed using db/db−/− mice to investigate ED, even though these animals have shown altered vasoreactivity consistent with impaired cavernosal relaxation and penile veno-occlusive disorder. The db/db−/− mice lack leptin receptors, and this deficiency contributes to the development of both diabetes and obesity. Therefore, these mice are widely considered an appropriate model for DM2, which has been used for the study of DM2-associated ED (Luttrell et al., 2008). In addition, db/db−/− mice develop hyperglycemia and hyperinsulinemia, the latter of which raises resting sympathetic output and contributes to impaired cavernosal relaxation (Anderson et al., 1991).

In this study, we examine the effect of BAY 41-2272 on relaxation of the CC from db/db−/− obese DM2 mice and their lean db−/− counterparts in response to vasodilatory agonists and the effects of the drug on markers of oxidative stress in these animals.

Materials and Methods

Animals. Male C5Bl/KsOlaHsd-leprdb/+ leprdb/+ mice (db/db−/−, with obesity and DM2 caused by a leptin-receptor mutation) and their lean, nondiabetic heterozygote (db+/+) and CL5 7bi6k littermates (14–16 weeks old; Harlan, Indianapolis, IN) were used in this study. The db/db−/− mice profile includes hyperinsulinemia, hyperglycemia, and obesity by 1–2 months of age. Animals were housed in accordance with the Georgia Regents University Animal Use for Research and Education Committee regulations. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. The mice were maintained in a 12-hour light/dark cycle room and were provided with standard rat chow and water ad libitum.

Glucose Levels and Plasma Lipids. Blood glucose levels were determined using an AccuCheck glucose meter (Roche Diagnostic Corporation, Indianapolis, IN) and glucose test strips in fasted mice. Plasma lipids were measured by commercially available colorimetric assays for cholesterol and triglycerides according to the manufacturer’s instruction (Cayman Chemical, Ann Arbor, MI).

Corpus Cavernosum Functional Studies. The mice were anesthetized with an isoflurane vaporizer, and cavernosal strips were obtained as described elsewhere (Toque et al., 2011). Concentration–response curves to BAY 41-2272, an sGC stimulator (10−8 to 10−5 M), acetylcholine (ACh; 10−8 to 10−5 M), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP; 10−8 to 10−5 M), an NO donor, were obtained in cavernosal strips contracted with phenylephrine (PE; 10−7 M, an α1 adrenergic receptor agonist). Also, both ACh and SNP curves were performed in the presence of BAY 41-2272 in different concentrations (10−7 M, 3 × 10−7 M, 10−7 M) after a 35-minute incubation.

In another set of experiments, cavernosal strips from lean db−/− and db/db−/− were incubated with guanethidine monosulfate (3 × 10−5 M) and atropine (10−5 M) to deplete the catecholamine stores and to block the muscarinic receptors in the presence or absence of different concentrations of BAY 41-2272 (10−8 M, 3 × 10−8 M, 10−7 M). After 35 minutes of incubation, the cavernosal strips were contracted with PE (10−7 M) and placed between two platinum electrodes connected to a Grass S88 stimulator (Astro-Med, West Warwick, RI). As previously described elsewhere, electrical field stimulation (EFS) was conducted at 20 V, 1-millisecond pulse width, and trains of stimuli lasting 10 seconds at varying frequencies (1–32 Hz) (Nunes et al., 2011).
Determination of Cyclic Nucleotide (GMP) Levels. To determine the cGMP contents in mice corpus cavernosum under experimental conditions, cavernosal strips were equilibrated for 20 minutes in warmed and oxygenated Krebs’ solution. Tissues were contracted with PE 10⁻⁵ M and then stimulated for 10 minutes with 1) BAY 41-2272 (10⁻⁷ M), ACh (10⁻⁶ M), or both; 2) BAY 41-2272 (10⁻⁷ M), EFS (4 Hz, 10 seconds), or both; and 3) BAY 41-2272 (10⁻⁷ M), SNP (10⁻⁶ M), or both. Next, the cavernosal strips were immediately collected by freezing the segments in liquid nitrogen. Some tissues were frozen after the incubation in vehicle to obtain baseline readings. Frozen cavernosal tissues were pulverized and homogenized in trichloroacetic acid (5% w/v) and then centrifuged at 1500 g for 10 minutes at 4°C. The trichloroacetic acid was extracted from samples with three washes of water-saturated ether. The weights of the dried pellets were used to standardize the different samples. We extracted and quantified cGMP using a cGMP enzyme immunoassay kit (Cayman Chemical). Preparation of tracer, samples, standards, and incubation with antibody were performed as described by the manufacturer’s instructions. Assays were performed in duplicate using different dilutions of samples.

Antioxidative Status. Antioxidative capacity was assessed by determining the total antioxidant status (TAS) in the plasma and urine from mice using a commercially available colorimetric assay kit (Cayman Chemical). Blood samples were collected from the abdominal aorta using an anticoagulant (citrate) and were centrifuged at 1000 g for 10 minutes at 4°C. The plasma collected was immediately frozen at −80°C for 48 hours. Urine samples were collected directly from the bladder using a syringe with a 27G needle and were immediately stored at −80°C for 48 hours.

Before performing the TAS assay, plasma and urine samples were diluted in a ratio of 1:20. Assays were performed according to the manufacturer’s instructions. Briefly, the TAS assay measures the antioxidative capacity of the sample by measuring the suppression of radical cation 2,2-azino-di-[3-ethylbenzthiazoline sulphonate] production.

Superoxide Measurements. ROS production was evaluated with measurements of superoxide. Cavernosal tissue was incubated for 1 hour, at 37°C in a 95% air, 5% CO₂ incubator, in the presence or absence of BAY 41-2272 (10⁻⁷ M). Tissues were then equilibrated in Dulbecco’s modified Eagle’s medium with no phenol red for 10 minutes 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 tissue sample was placed in the incubator for 1 hour at 37°C. The reaction medium was removed, and reduction of the amount of cytochrome c was determined at 550 nm and converted to millimoles of O₂⁻ using a ΔE₅₅₀nm of 2.1 mmol L⁻¹ cm⁻¹ as the extinction coefficient. The reduction of cytochrome c, which was inhibited with superoxide

### TABLE 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>db/+</th>
<th>db/db⁻⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>28.3 ± 0.7</td>
<td>50.1 ± 0.6*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>113.9 ± 20.9</td>
<td>542.1 ± 30.3*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>22.3 ± 0.7</td>
<td>31.9 ± 4.5*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>60.4 ± 2.6</td>
<td>94.5 ± 6.8*</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>16.0 ± 0.6</td>
<td>45.5 ± 6.6*</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>39.9 ± 2.4</td>
<td>42.6 ± 1.7</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with the db/+ group.

**Fig. 1.** (A) Concentration-response curves to BAY 41-2272 (10⁻⁸ to 10⁻⁵ M) in CC from db/+ and db/db⁻⁻ mice contracted with PE (10⁻⁵ M). (B) Impaired ACh-induced relaxation was observed in CC from db/db⁻⁻ compared with lean db/+ mice. Enhancement of responses to ACh-induced relaxation was observed in the presence of BAY 41-2272 in CC from (C) db/+ and (D) obese, diabetic db/db⁻⁻ mice. Data represent the mean ± S.E.M. of six experiments. *P < 0.05, **P < 0.01 versus db/+.
of the following composition was used: 130 mM NaCl, 14.9 mM NaHCO₃, 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄·7H₂O, 1.6 mM CaCl₂. We purchased 2H₃O₂ and 0.026 mM EDTA, atropine, PE, ACh, SNP, apocynin, and U46619 (9-11-dideoxy-11α,9β-epoxymethano-prostaglandin F₂α) from Santa Cruz Biotechnology (Santa Cruz, CA). After incubation with secondary antibodies, we detected signals by enhanced chemiluminescence autoradiography. The bands were quantified by densitometric scanning of film images using UN-SCAN-IT software (Silk Scientific, Orem, UT). The results were normalized to β-actin protein and expressed as arbitrary units. The antibody used to probe for β-actin was acquired from Sigma-Aldrich (1:2000).

**Western Blotting.** Proteins (20 μg) extracted from cavernosal preparations were separated by electrophoresis on a 10% SDS-polyacrylamide precast gel and transferred to a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline/Tween for 1 hour at 24°C. Membranes were incubated overnight at 4°C with the primary antibodies anti-gp91phox 1:500, anti-p22phox 1:500, and anti-p47phox 1:500, purchased from Axxora Life Sciences (San Diego, CA). A physiologic salt solution (137 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 8.1 mM Na₂HCO₃, 0.27 mM NaH₂PO₄·2H₂O, pH 7.4) was also used to rinse the tissues before use.

**Data Analysis and Statistics.** Experimental values of relaxation or contraction were calculated relative to the maximal changes from the steady-state tension. In protocols where EFS was studied, each measurement of relaxation was adjusted for minor fluctuations (during the course of the experiment) in the steady-state tension. Data are shown as the percentage of relaxation of n experiments, expressed as the mean ± S.E.M. Curves were fitted to all the data using nonlinear regression. One-way analysis of variance and Student's t test were used to evaluate and compare the results. The Bonferroni correction was employed for multiple comparisons. P < 0.05 was considered statistically significant. Calculations were performed with Prism, version 5.00 (GraphPad Software, San Diego, CA).

**Results**

**Glucose and Lipid Profile of db/+ and db/db−/− Mice.** As expected, db/db−/− mice displayed higher body weight and glucose levels than db/+ lean mice (50.1 ± 0.6 versus 28.3 ± 0.7 g, respectively, and 542.1 ± 30.3 versus 113.9 ± 20.9 mg/dl, n = 6 group). These obese mice also showed elevated levels of triglycerides (31.9 ± 4.5 versus 22 ± 0.7 mg/dl⁻¹), total cholesterol (94.5 ± 6.8 versus 60.4 ± 2.6 mg/dl⁻¹), and high-density lipoprotein cholesterol (45.5 ± 6.6 versus 16.0 ± 0.6 mg/dl⁻¹) as described in Table 1. There were no differences in low-density lipoprotein cholesterol levels (42.6 ± 6.6 versus 39.9 ± 2.4 mg/dl⁻¹).

**BAY 41-2272 Relaxes Diabetic CC and Attenuates Endothelium-Dependent Cavernosal Dysfunction.** To evaluate the independent relaxing activity of BAY 41-2272, concentration–response curves (10⁻⁸ to 10⁻⁵ M BAY 41-2272) were ascertained via cavernosal strips contracted with PE (10⁻⁵ M). BAY 41-2272 potently relaxed CC from diabetic/obese (db/db−/−) and lean (db/+ ) mice (Fig. 1A). Vascular endothelial function was indirectly determined by relaxation to ACh in the CC tissue. In PE-contracted CC strips, the cumulative addition of ACh (10⁻⁸ to 10⁻⁵ M) produced lower relaxation in db/db−/− mice compared with age-matched db/+ lean mice (Fig. 1B). However, responses to ACh were enhanced in the presence of BAY 41-2272 at different concentrations (10⁻⁸ to 10⁻⁵ M) in both lean (Fig. 1C) and db/db−/− diabetic/obese mice (Fig. 1D). As observed in Fig. 1D, BAY 41-2272 markedly reduced the cavernosal endothelial dysfunction in obese, diabetic db/db−/− mice.

**The Combination of BAY 41-2272 and SNP Effectively Relaxes Diabetic CC.** The endothelium-independent relaxation induced by SNP (NO donor) showed no difference between strains (pEC50; 6.65 ± 0.06 versus 6.49 ± 0.02; Emax: 86% ± 3% and 93% ± 4%, for lean and db/db−/− mice, respectively, n = 6) (Fig. 2A). In spite of this, the potency values (pEC50) observed in response to SNP were clearly increased by pretreatment with 10⁻⁵ M, 3 × 10⁻⁶ M, and 10⁻⁷ M BAY 41-2272 in CC from db/+ and db/db−/− mice. Data represent the mean ± S.E.M. of four experiments. *P < 0.05, **P < 0.01, compared with the respective CC control. CTL, control.
Fig. 3. (A) Electrical field stimulation (EFS)–induced nitrergic relaxation (1–32 Hz) in CC from db/+ and diabetic/obese db/db−/− mice. (B) Magnitude and (C) duration of EFS-induced relaxations were increased in the presence of 10⁻⁸ M, 3 × 10⁻⁸ M, and 10⁻⁷ M BAY 41-2272. (D) Representative original traces of 32 Hz in CC from db/+ and db/db−/− mice in the absence or presence of 10⁻⁸ M, 3 × 10⁻⁸ M, and 10⁻⁷ M BAY 41-2272. Data represent the mean ± S.E.M. of five experiments. *P < 0.05, **P < 0.01, compared with the respective CC control. CTL, control.
relaxation was performed in strips contracted with PE using EFS (1–32 Hz). Diabetic/obese mice (db/db) showed reduced nitricergic cavernosal relaxation compared with lean (db/+) at various frequencies (1–32 Hz, n = 6) (Fig. 3A). However, the amplitude of relaxation induced by EFS in CC from diabetic mice was significantly increased in the presence of BAY 41-2272 (Fig. 3B). The duration of nitrogliceric relaxation was similar in both strains and was enhanced in the presence of BAY 41-2272 (Fig. 3, C and D). The highest level of cavernosal relaxation in the presence of this compound was observed at a concentration of 10^{-7} M.

**Levels of cGMP Are Increased by BAY 41-2272 in Penile Tissue of Diabetic Mice.** In the presence of ACh (10^{-8} M) or SNP (10^{-6} M), the cGMP levels were equally increased in both lean and db/db diabetic/obese mice (Fig. 4, A and C, respectively), compared with basal values. However, cGMP levels after EFS (4 Hz) stimulation of CC were higher in lean db/+ mice than in db/db diabetic/obese mice (Fig. 4B). In CC treated with BAY 41-2272 (10^{-7} M), the cGMP was significantly increased, above control levels in both strains (Fig. 4, A–C). In strips incubated with a combination of BAY 41-2272 with EFS and BAY 41-2272 with SNP (Fig. 4, B and C, respectively), the resulting intracellular cGMP levels were markedly above the sum of their effects alone (P < 0.01) in both strains. The cGMP levels were increased in CC incubated with BAY 41-2272 and ACh in both lean db/+ and obese/db/db diabetic/obese mice, but not above the sum of their effects alone (Fig. 4A). There was no statistically significant difference in the basal cGMP content in CC between diabetic/obese (db/db -/−) and lean (db/+ +) mice (Fig. 4, A–C, respectively).

**Inhibitory Effect of BAY 41-2272 on NADPH Expression in Diabetic Cavernosal Tissue.** When the expression of NADPH oxidase subunits (gp91phox, p22phox, and p47phox) was assessed, cavernosal tissue from diabetic mice (db/db -/−) showed augmented expressions for all three subunits compared with lean (db/) mice (Fig. 5). No changes were observed in the expression of the subunits p67phox and p40phox (data not shown). To evaluate whether BAY 41-2272 affects NADPH expression in diabetic db/db -/− mice, we incubated cavernosal strips with two different BAY 41-2272 concentrations (10^{-8} and 10^{-7} M). In these diabetic tissues, BAY 41-2272 noticeably reduced the expression of gp91phox (Fig. 5D), p22phox (Fig. 5E), and p47phox (Fig. 5F) after 8 hours of incubation.

**Increased Superoxide Production in Diabetic Mice (db/db -/−) Is Prevented by BAY 41-2272.** The oxidative stress in diabetic and obese mice (db/db -/−) was confirmed by measuring the TAS in the plasma and urine (Fig. 6A). In the plasma, the TAS levels were significantly diminished in diabetic mice compared with lean (0.46 ± 0.1 versus 1.7 ± 0.2 mM, diabetic db/db -/− versus lean db/+, respectively) as well as in urine (3.69 ± 0.4 versus 5.8 ± 0.8 mM, diabetic db/db -/− versus lean db/+, respectively). We measured superoxide production in cavernosal tissue from diabetic mice (db/db -/−) in the presence or absence of BAY 41-2272 (10^{-7} M). Higher superoxide production was observed in cavernosal strips from diabetic compared with lean mice (7.05 ± 0.71 versus 3.82 ± 0.47, db/db -/− versus db/+, respectively). Incubation with BAY 41-2272 significantly decreased superoxide production in diabetic CC (7.05 ± 0.71 versus 4.50 ± 0.46, nontreated versus treated, respectively) (Fig. 6B).

**Discussion**

Our data showed that in diabetic, obese (db/db -/−) mice BAY 41-2272 ameliorated impaired endothelial and nitricergic cavernosal relaxation by elevating the intracellular cGMP production in cavernosal tissue from diabetic mice (db/db -/−)
concentration, preventing elevated expression of NADPH oxidase enzyme subunits, and decreasing superoxide formation. Although the pathogenesis of ED in diabetes is multifactorial, vascular dysfunction is a major contributor to the high incidence of ED in men with diabetes (Chu and Edelman, 2002).

Previous studies have shown that alteration of the cGMP-NO pathway among diabetic men with impaired vascular relaxation is related to endothelial dysfunction (Angulo et al., 2010). Although PDE-5 inhibitors are often the preferred therapy for most men with vasculogenic ED, the efficacy of...
Diabetic mice, (including db/db)

In support of this, many studies using different models of nitrergic NO release is impaired (Moore and Wang, 2006). Andersson, 2011). In diabetic patients it is thought that And in diabetic patients, the endothelium-dependent response is impaired. However, this result was prevented after incubation with BAY 41-2272 (Fig. 3, B and C). More importantly, it consistently extended the magnitude and duration of relaxation (Fig. 3, B and C) in diabetic/obese db/db−/− and lean db/+ CC. Considering that decreased NO availability is a common factor in diabetes-related ED, prolonged cavernosal relaxation in an in vivo situation would be extremely beneficial, leading to or improving the quality of an erection. In addition, this result supports the concept that BAY 41-2272 interacts with endogenous NO (Teixeira et al., 2006c, 2007).

The level of accumulated cGMP is an indicator of NO production and sGC activation. In diabetic patients a reduced content of cGMP was demonstrated in the CC (Angulo et al., 2009). In contrast to classic NO donors, BAY 41-2272 directly stimulates sGC independently of NO and increases the NO sensitivity of sGC, generating significant amounts of cGMP. This increase in cGMP production in the presence of BAY 41-2272 was observed in both db/+ and db/db−/− CC in response to ACh, SNP, or EFS (Fig. 4). However, there was no statistically significant difference between strains in the relative increase in response to ACh or SNP.

On the other hand, it has been suggested that neuronal stimulation, not cholinergic stimulation, is vital in the erectile process. Cavernosal tissue from diabetic mice incubated with BAY 41-2272 and SNP resulted in much higher cGMP production compared with the effect of each drug by itself (Fig. 4C). This conclusion is reinforced by results obtained in rat mesenteric artery (Teixeira et al., 2006c, 2007), basilar artery (Teixeira et al., 2006b), and anoccygeous muscle (Teixeira et al., 2006a). A similar result was observed when tissue was coincubated with BAY 41-2272 for EFS but not ACh. We believe that NO production from endothelial NOS triggered in response to ACh is responsible for sustaining normal penile erection, whereas NO from nNOS triggered by EFS is responsible for initiating and maintaining the normal erection process. Therefore, NO from nitrergic nerves plays a major role in an erection, which can explain why the presence of BAY made a significant difference when cGMP production was induced by EFS (Fig. 4B) but not ACh (Fig. 4A).

Diabetes and ED are vascular diseases related to oxidant status (Jin and Burnett, 2008). One of the potential mechanisms involved in endothelial dysfunction, not only in diabetes but also in vascular diseases in general, includes increased levels of oxygen free radicals such as superoxide anion. It has been shown that blood vessels of diabetic patients exhibit excessive superoxide anion production (Guzik et al., 2002). Increased levels of superoxide anion quench NO, leading to impaired vascular function and ED (Burnett et al., 2006). Additionally, superoxide oxidizes NO cofactors, leading to uncoupling of this enzyme and further increases in ROS production.

NADPH oxidase is a major source for ROS formation in the vascular wall (Griendling et al., 2000). It has been suggested that NADPH oxidase–derived ROS are produced at low levels in the penis under normal physiologic conditions. However, enhanced activity of this enzyme leads to elevated ROS production in isolated cavernosal smooth muscle cells.
(Koupparis et al., 2005). Many studies have shown that NADPH oxidase is upregulated in conjunction with enhanced ROS and severe ED in hypertensive rats, hypercholesterolemic rabbits, diabetic mice, and rat models of sleep apnea (Shukla et al., 2005, 2009; Jin et al., 2008; Liu et al., 2012). In addition to NADPH oxidase, other ROS-producing enzymes may be responsible for the oxidative stress observed in the cavernous tissue during ED, such as the mitochondrial respiratory chain, xanthine oxidase, or uncoupled NOS. Although the exact role of NADPH oxidase in ED is still poorly understood (compared with other vascular diseases) it has been suggested this enzyme plays an important role in the development of ED (Jin and Burnett, 2008). However, no available evidence indicates which isoform of NADPH oxidase is prevalent in penile tissue, and there is no study investigating this enzyme in CC from obese, diabetic db/db mice.

Our study assessed the effect of BAY 41-2272 on NADPH oxidase subunits in CC from diabetic db/db mice. Our results showed that BAY 41-2272 prevented the enhanced expression of subunits gp91phox, p22phox, and p47phox (Fig. 5, A–C) observed in CC of diabetic db/db–/– mice (Fig. 5, D–F). These data suggest that BAY 41-2272 may be acting not only in a direct manner to increase sCG activity (explaining the fast effect of BAY 41-2272 on CC relaxation responses) but also in an indirect manner by decreasing superoxide production levels, likely via modulating NADPH oxidase expression (explaining the long-term incubation effects of BAY 41-2272). No difference was observed in the subunits p67phox and p47phox (Fig. 5, D–F). These findings are consistent with a previous report that NADPH oxidase–dependent superoxide generation is significantly increased after incubating mouse CC with U46619, a stimulator of superoxide formation. This increase in superoxide production was reversed by BAY 41-2272 via decreased protein expression of NADPH oxidase subunits (Teixeira et al., 2007).

Antioxidants terminate the detrimental effect of free radicals by preventing the formation of radicals, by scavenging them, or by promoting their decomposition. Therefore, we evaluated the TAS, a measure of indirect information on oxidative stress. Lower levels of antioxidants (expressed as TAS) were found in plasma and urine from obese, diabetic/db/db–/– mice compared with lean mice (Fig. 6A), supporting that oxidative stress is increased in penile tissue from these diabetic animals. Additionally, our data showed that augmented superoxide production in diabetic/db/db–/– mice cavernosal tissue was inhibited by incubation with BAY 41-2272 (Fig. 6B). Although the protein expression data support the idea that NADPH oxidase may be responsible for the increased superoxide production and the beneficial effects of BAY 41-2272 in obese, diabetic/db/db–/– penile tissue, these results cannot exclude the potential contribution of ROS from other sources. Determining the exact relative contribution to NADPH oxidase, xanthine oxidase, NOS, mitochondria, and other ROS producers will be the focus of a future study.

Taken together, our data reveal that decreased cGMP levels, increased superoxide production, and expression of NADPH oxidase subunits displayed by obese, diabetic/db/db–/– mice contribute to the impaired cavernosal function observed in these animals. These negative effects were prevented or decreased in the presence of BAY 41-2272, making this NO-independent stimulator of sGC an attractive alternative therapy for treatment of diabetic patients.

Acknowledgments
The authors thank Dr. Theodora Szasz, Michelle Lashley, and Inger Stallmann for helping revise the final version of this manuscript, and Dr. Richard L. Moss for supporting the research of K.P.N.  

Authorship Contributions
Participated in research design: Webb, Teixeira.  
Conducted experiments: Teixeira, Nunes.  
Performed data analysis: Teixeira, Nunes, Toque, Priviero.  
Wrote or contributed to the writing of the manuscript: Nunes.

References

Nunes et al.


Address correspondence to: Dr. Kenia Pedrosa Nunes, University of Wisconsin, Department of Cell and Regenerative Biology, 1111 Highland Avenue, Room 8548, WIMR, Madison, WI 53719. E-mail: keniapedrosa@gmail.com.