Beneficial effect of the soluble guanylyl cyclase stimulator BAY 41-2272 on impaired penile erection in db/db−/− type II diabetic and obese mice

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List of non-standard abbreviations:

ACh- acetylcholine
BAY 41-2272- (5-cyclopropyl-2-[1-2-fluoro-benzyl]-1H-pyrazolo[3,4-b]pyridine-3-yl)pyrimidin-4ylamine
CC- corpus cavernosum
cGMP- cyclic guanosine monophosphate
DM2- Type 2 diabetes mellitus
EC₅₀- 50% effective concentration
ED- erectile dysfunction
EFS- electrical field stimulation
GTP- guanosine 5’-triphosphate
NANC- non-adrenergic, non-cholinergic
NADPH- nicotinamide-adenine dinucleotide phosphate
NO- nitric oxide
NOS- nitric oxide synthase
PDE-5- phosphodiesterase type 5
PE- phenylephrine
ROS- reactive oxygen species
S.E.M.- standard error of mean
sGC- soluble guanylyl cyclase
TAS- total anti-oxidants
v/v- volume/volume

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3- 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 (PDE-5) inhibitors. This study investigated whether a soluble guanylyl cyclase (sGC) stimulator, BAY 41-2272, is effective in improving impaired corpus cavernosum (CC) relaxation in obese, DM2 mice by reducing oxidative stress. Adult db/db-/- mice or their lean 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^-5M) potently relaxed CC from db/+ or db/db/- mice in a similar manner. BAY 41-2272 significantly enhanced both endothelium-dependent and nitrergic 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^-7M). BAY 41-2272 increased cGMP levels and potentiated relaxation responses to exogenous NO in CC. Total antioxidant status (TAS) was reduced in plasma and urine, while expression of vascular NADPH oxidase subunits (gp91phox, p22phox, p47phox) was increased in the CC of 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 db/db-/- mice by increasing cGMP and augmenting antioxidant status, therefore this drug is a potential novel candidate to treat ED.
4- Introduction

Diabetic men are three times more likely to suffer from ED than non-diabetic 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 erectile dysfunction (ED) (Bacon et al., 2006; Giugliano et al., 2010) because of their deleterious effects on the vasculature (Tamler, 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 which 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 (Gratzke et al., 2010; Andersson, 2011). The gaseous NO molecule, with major vasodilatory effects, is derived from neuronal and endothelial nitric oxide synthase (nNOS and eNOS). Once released from nitrergic nerve endings and the endothelium lining the arteries supplying the penis and the cavernosal sinusoid spaces, it 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, 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 therapeutical 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; Lasker et al., 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 (Evgenov et al., 2006).
BAY 41-2272 (5-cyclopropyl-2-[1-2-fluoro-benzyl]-1H-pyrazolo[3,4-b]pyridine-3-yl)pyrimidin-4yl amine) is a sGC stimulator that has been shown to produce anti-proliferative and vasodilatory effects (Evgenov et al., 2006), as well as to potentiate erectile responses (Bischoff et al., 2003) and relax the CC of human and animal (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 regarding 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 5 subunits, including p47phox (Lassegue and Clempus, 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 (O_2^-) formation in the vasculature interferes with the complex mechanisms underlying normal penile erection. The excessive production of O_2^- 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 (STZ)-induced diabetic rats (Cheah et al., 2002). These data suggest 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<sup>−/−</sup> mice to investigate ED, even though these animals have showed altered vasoreactivity consistent with impaired cavernosal relaxation and penile venoocclusive disorder. The db/db<sup>−/−</sup> mice lack the leptin receptors and this deficiency contributes to the development of both diabetes and obesity. Therefore, these mice are widely considered an appropriate model for type 2 diabetes, which has been used for the study of type 2 diabetes-associated ED (Luttrell et al., 2008). In addition, db/db<sup>−/−</sup> 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<sup>−/−</sup> obese, type 2 diabetic mice and their lean db/<sup>+/+</sup> counterparts in response to vasodilatory agonists, and the effects of the drug on markers of oxidative stress in these animals.
5- Material and Methods

Animals

Male C5BL/KsOlaHsd-lepr\^{db}/lepr\^{db} mice (db/db\textsuperscript{-/-}, mice with obesity and type 2 diabetes caused by a leptin receptor mutation) and their lean, non-diabetic heterozygote (db/+ ) and CL5 7/bl6k littermates (14-16 weeks old, Harlan, Indianapolis, IN, USA) were used in this study. The db/db\textsuperscript{-/-} 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. Experiments have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institute of Health. The mice were maintained in a 12-hour light-dark cycle room and were provided with standard rat chow and water \textit{ad libitum}.

Glucose levels and Plasma lipids

Blood glucose levels were determined using an AccuCheck glucose meter (Roche Diagnostic Corporation, Indianapolis, IN, USA), 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 Isoflurane vaporizer and cavernosal strips were obtained as described elsewhere (Toque et al., 2010). Concentration–response curves to BAY 41-2272, a soluble guanylyl cyclase (sGC) stimulator (BAY, 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^{-5}M, an \alpha_1 adrenergic receptor agonist). Also, both ACh and SNP curves were performed in the presence of BAY 41-2272 in different concentrations (10^{-8}M, 3x10^{-8}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 monosulphate (3x10^{-3}M) and atropine (10^{-6}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, 3x10^{-8}M, 10^{-7}M). After 35 minutes of incubation cavernosal strips were contracted with PE (10^{-5}M) and placed between two platinum electrodes connected to a Grass S88 stimulator (Astro-Med Industrial Park, RI, USA). As previously described, EFS was conducted at 20 V, 1 ms pulse width and trains of stimuli lasting 10s 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; 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 following the incubation in vehicle to obtain baseline readings. Frozen cavernosal tissues were pulverized and homogenized in trichloroacetic acid (TCA; 5% w/v) and then centrifuged at 1500g for 10 minutes at 4°C. The TCA was extracted from samples with three washes of water-saturated ether. The weights of the dried pellets were used to standardize the different samples. cGMP was extracted and quantified using a cGMP enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). 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.

**Anti-oxidative status**

Anti-oxidative capacity was assessed by determining the total anti-oxidants (TAS) in the plasma and urine from mice using commercially available colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Blood samples were collected from the abdominal aorta using an anticoagulant (citrate) and centrifuged at 1000g 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 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 anti-oxidative capacity of the sample by measuring the suppression of radical cation 2,2’-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) production.

**Superoxide measurements**

Reactive oxygen species production was evaluated with measurements of superoxide. Cavernosal tissue was incubated for 1h, at 37ºC in a 95% air, 5% CO₂ incubator, in the presence or absence of BAY 41-2272 (10⁻⁷M). Tissues were then equilibrated in Dulbecco’s modified Eagle’s medium with no phenol red for 10min at 37ºC in the incubator. Next, 20 mM horseradish cytochrome c (Sigma Aldrich, St. Louis, MO), with or without 500U/ml copper-zinc superoxide dismutase, was added, and the tissue sample was placed in the incubator for 1h at 37ºC. The reaction medium was removed; 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⁻¹cm⁻¹ as the extinction coefficient. The reduction of cytochrome c, which was inhibited with superoxide dismutase, reflected actual O₂⁻ release. Tissue was rinsed in phosphate-buffered saline, lysed with 0.1% (v/v) Triton x-100, and total protein content was measured. The results were expressed as millimoles of O₂⁻ per milligram of protein per hour.
Western Blotting

Proteins (20 μg) extracted from cavernosal preparations were separated by electrophoresis on a 10% SDS-polyacrylamide pre-cast gel and transferred to a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline/Tween for 1h at 24°C. Membranes were incubated with primary antibodies (anti-gp91phox 1:500, anti p22phox 1:500, and p47phox 1:500 purchased from Santa Cruz Biotechnology, Inc., CA, catalog number sc-5827, sc-14015 and sc-7660 respectively) overnight at 4°C. After incubation with secondary antibodies, signals were detected by enhanced chemiluminescence autoradiography. The bands were quantified by densitometric scanning of film images using UN-SCAN-IT software (Silk Scientific, Inc.). 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, catalog number A1978).

Drugs and Solutions

The compound BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-qaPyrazolo[3,4-b]pyridine-3-yl]-pyrimidin-4-ylamine) was obtained from Axxora Life Sciences, Inc (San Diego, CA). Physiological salt solution 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 CaCl₂·2H₂O, and
0.026 mM EDTA (ethylenediaminetetraacetic acid). Atropine, PE, ACh, SNP, apocynin and U46619 were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and guanethidine monosulfate was purchased from USP (Rockville, MD, USA). All reagents used were of analytical grade. Stock solutions were prepared in deionized water and stored in aliquots at -20°C; dilutions were made up immediately before use.

Data analysis and Statistics

Experimental values of relaxation or contraction were calculated relative to the maximal changes from the contraction produced by PE (taken as 100% in each tissue). The pEC$_{50}$ value was determined as –log of the molar concentration to produce 50% of the maximal relaxation in PE-contracted tissues. In protocols where EFS was studied, each measurement of relaxation was normalized to the calculated net tension just before stimulation in order to adjust for minor fluctuations (during the course of the experiment) in the steady-state tension. Data were 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 (ANOVA) 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 using Prism, version 5.00 (GraphPAD Software Inc., SanDiego, CA).
6- 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 “vs.” 28.3 ± 0.7g respectively, and 542.1 ± 30.3 “vs.” 113.9 ± 20.9 mg dl⁻¹, n=6 group). These obese mice also showed elevated levels of triglycerides (31.9 ± 4.5 “vs.” 22 ± 0.7 mg dl⁻¹), total cholesterol (94.5 ± 6.8 “vs.” 60.4 ± 2.6 mg dl⁻¹) and HDL-cholesterol (45.5 ± 6.6 “vs.” 16.0 ± 0.6 mg dl⁻¹) as described in Table 1. There were no differences in LDL-cholesterol levels (42.6 ± 6.6 “vs.” 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 10⁻⁵M BAY 41-2272) were performed in cavernosal strips contracted with PE (10⁻⁵M). BAY 41-2272 potently relaxed CC from diabetic/obese (db/db⁻/⁻) and lean (db/+ ) mice (Figure 1A). Vascular endothelial function was indirectly determined by relaxation to ACh in the CC tissue. In PE contracted CC strips, cumulative addition of ACh (10⁻⁸ to 10⁻⁵M) produced lower relaxation in db/db⁻/⁻ mice compared to aged-matched db/+ lean mice (Figure 1B). However, responses to ACh were enhanced in the presence of BAY 41-2272 at different concentrations (10⁻⁸ to 10⁻⁷M) in both lean (Figure 1C) and db/db⁻/⁻ diabetic/obese
mice (Figure 1D). As observed in figure 1D, BAY 41-2272 markedly reduced the cavernosal endothelial dysfunction in obese, diabetic mice db/db⁻/⁻.

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 “vs.” 6.49 ± 0.02 and Emax: 86 ± 3% and 93 ± 4%, for lean and db/db⁻/⁻, respectively, n=6) (Figure 2A). In spite of this, the potency values (pEC₅₀) observed in response to SNP were clearly increased by pre-treatment with BAY 41-2272 to relax CC from diabetic (db/db⁻/⁻) as well as lean (db/+ ) mice (Figure 2B). The maximum relaxation responses reached by SNP were used to calculate the pEC₅₀ values in CC tissues from db/db⁻/⁻ and control mice.

**BAY 41-2272 improves nitrergic-induced relaxation in diabetic mice.**

Because NO produced by nNOS and released from non-adrenergic, non-cholinergic (NANC or nitrergic) nerves is the most important mediator of cavernosal relaxation, nitrergic relaxation response was evaluated. Frequency-dependent relaxation was performed in strips contracted with PE using electrical field stimulation (EFS, 1-32 Hz). Diabetic/obese mice (db/db⁻/⁻) showed reduced nitrergic cavernosal relaxation compared to lean (db/+ ) at various frequencies (1 to 32Hz, n=6) (Figure 3A). However, the amplitude of relaxation induced by EFS, in CC from diabetic mice, was significantly increased in the presence of BAY 41-2272 (Figure 3B). The duration of nitrergic relaxation was similar in both strains.
and enhanced in the presence of BAY 41-2272 (Figure 3C and 3D). The highest level of cavernosal relaxation in the presence of this compound was observed at a concentration of $10^{-7}\text{M}$.

*Levels of cGMP are increased by BAY 41-2272 in penile tissue of diabetic mice*

In the presence of ACh ($10^{-6}\text{M}$) or SNP ($10^{-6}\text{M}$), the cGMP levels were equally increased, in both lean and db/db$^{-/-}$ diabetic/obese mice (Figure 4A and 4C respectively), compared to basal values. However, cGMP levels following EFS (4Hz) stimulation of CC were higher in lean db+ mice than in db/db$^{-/-}$ diabetic/obese mice (Figure 4B). In CC treated with BAY 41-2272 ($10^{-7}\text{M}$) the cGMP was significantly increased, above control levels, in both strains (Figure 4A, 4B and 4C). In strips incubated with a combination of BAY 41-2272 with EFS and BAY 41-2272 with SNP (Figure 4B and 4C respectively), the resulting intracellular cGMP levels were markedly above the sum of their effects alone ($^{*}\text{P}<0.01$) in both strains. The cGMP levels were increased in CC incubated with BAY 41-2272 and ACh in both lean db+ and obese/diabetic db/db$^{-/-}$ mice, but not above the sum of their effects alone (Figure 4A). There was no statistical difference in the basal cGMP contents in CC between diabetic/obese (db/db$^{-/-}$) and lean (db$^{+/+}$) mice (Figure 4A, 4B and 4C respectively).
Inhibitory effect of BAY 41-2272 on NADPH expression in diabetic cavernosal tissue

Expression of NADPH oxidase subunits (gp91phox, p22phox, and p47phox) was assessed and cavernosal tissue from diabetic mice (db/db⁻/⁻) showed augmented expressions for all three subunits compared to lean (db/+⁻) mice (Figure 5A, 5B and 5C respectively). 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, cavernosal strips were incubated with two different BAY 41-2272 concentrations (10⁻⁸ and 10⁻⁷M). In these diabetic tissues, BAY 41-2272 noticeably reduced the expression of gp91phox (Figure 5D), p22phox (Figure 5E) and p47phox (Figure 5F) after 8h 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 total antioxidant status (TAS) in the plasma and urine (Figure 6A). In the plasma, TAS levels were significantly diminished in diabetic mice compared to lean (0.46±0.1 vs. 1.7±0.2 millimoles/L, diabetic db/db⁻/⁻ and lean db⁻/⁻ respectively), as well as in urine (3.69±0.4 vs. 5.8±0.8 millimoles/L, diabetic db/db⁻/⁻ and lean db/+ respectively). Superoxide production was measured in cavernosal tissue from diabetic mice (db/db⁻/⁻) in the presence or absence of BAY 41-2272 (10⁻⁷M). Higher superoxide production was observed in cavernosal strips from diabetic compared to lean mice (7.05±0.71 vs. 3.82±0.47, db/db⁻/⁻ and db/+ respectively).
respectively). Incubation with BAY 41-2272 significantly decreased superoxide production in diabetic CC (7.05±0.71 vs. 4.50±0.46, non-treated and treated, respectively) (Figure 6B).
7- Discussion

Our data showed that in diabetic, obese (db/db⁻/⁻) mice, BAY 41-2272 ameliorated the impaired endothelial and nitrergic cavernosal relaxation by elevating intracellular cGMP 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 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 these drugs is decreased in diabetic patients (Isidro, 2012), underlying the need for new treatment options. It has been shown that BAY 41-2272 is a potent relaxing agent in many tissues (Priviero et al., 2005; Teixeira et al., 2006a; Toque et al., 2008), including the CC. However, this compound has not yet been investigated in diabetes-associated ED.

In this study, BAY 41-2272 was shown to relax CC of diabetic and control mice in a similar manner (Figure 1A). In diabetic db/db⁻/⁻ mice relaxation evoked by ACh suggested an impaired endothelium-dependent response. However, this effect was prevented after incubation with BAY 41-2272 (Figure 1D). In figure 2B, our data showed that BAY 41-2272 potentiates SNP-induced relaxation in diabetic db/db⁻/⁻ mice. Corroborating this result, in wild type mice, endothelium-independent
relaxation response curves of CC induced by SNP were shifted to the left as a function of increasing concentrations of BAY 41-2272 (Teixeira et al., 2007).

The erectile response is considered to be initiated by NO release from nitrergic nerves in the CC (Toda et al., 2005; Andersson, 2011). In diabetic patients it is thought that nitrergic NO release is impaired (Moore and Wang, 2006). In support of this, many studies using different models of diabetic mice, (including db/db^{-/-}) have shown inefficient nitrergic nerve-mediated relaxations in CC (Carneiro et al., 2008; Nunes et al., 2011; Toque et al., 2013a; Toque et al., 2013b). Our data, in tandem with these studies, showed decreased nitrergic-induced cavernosal relaxation in db/db^{-/-} mice. Nevertheless, BAY 41-2272 improved the magnitude of nitrergic-induced relaxations by EFS (Figure 3B and 3C). More importantly, it consistently extended the magnitude and duration of relaxation (Figure 3B and 3C) 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; Teixeira et al., 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 classical 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 (Figure 4). However, there was no statistical difference between strains in the relative increase in response to ACh or SNP. On the other hand, it has been suggested that neuronal stimulation, and not cholinergic stimulation, is vital in the erectile process. Cavernosal tissue from obese, diabetic mice co-incubated with BAY 41-2272 and SNP resulted in much higher cGMP production compared to the effect of each drug by itself (Figure 4C). This conclusion is reinforced by results obtained in rat mesenteric artery (Teixeira et al., 2006c), basilar artery (Teixeira et al., 2006b) and anococcygeus muscle (Teixeira et al., 2006a). A similar result was observed when tissue was co-incubated with BAY 41-2272 for EFS, but not ACh. We believe that NO production from eNOS 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 in the presence of BAY there was significant difference when cGMP production is induced by EFS (Figure 4B), but not ACh (Figure 4A). Diabetes, as well as 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 NOS cofactors, and leads to uncoupling of this enzyme and further increases 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 physiological 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; Jin et al., 2008; Shukla et al., 2009; Liu et al., 2012). In addition to NADPH oxidase, other ROS-producing enzymes may be responsible for the oxidative stress observed in the cavernousum 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 to 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.

In this study, the effect of BAY 41-2272 on NADPH oxidase subunits in CC from diabetic db/db −/− mice was assessed. Our results showed that BAY 41-2272
prevented the enhanced expression of subunits gp91phox, p22phox, and p47phox (Figure 5A, 5B and 5C) observed in CC of diabetic db/db mice (Figure 5D, 5E and 5F). 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 p40phox. These findings are consistent with a previous report that states 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, scavenging them, or by promoting their decomposition. Therefore, we evaluated the total antioxidant status (TAS), a measure which provides 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 to lean mice (Figure 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 (Figure 6B). Although the protein expression data supports 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, XO, NOS, mitochondria and other ROS producers will be the focus of a future study.

Taken together, our data revealed 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 ED in diabetic patients.
8- Conflict of interest

The authors declare no conflict of interest.
9- **Acknowledgments:**

Dr. Cleber E. Teixeira helped design, perform and interpret the experiments presented in this manuscript. Unfortunately, Dr. Teixeira passed away before the publication of this manuscript, to which he was an essential contributor. We thank Dr. Theodora Szasz, Ms. Michelle Lashley and Ms. Inger Stallmann for helping revise the final version of this manuscript. We thank you Dr. Richard L. Moss for supporting Dr. Nunes research.
10-Authorship Contributions

Research design: Webb, Teixeira

Conducted experiments: Teixeira, Nunes

Performed data analyses: Teixeira, Nunes, Toque, Priviero

Wrote Manuscript: Nunes

Review and Approval: Nunes, Toque, Priviero and Webb
11- References


Footnotes:

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12- Legends for Figures

Figure 1. Concentration-response curves to BAY 41-2272 (10^{-8} to 10^{-5} M; panel A) in CC from db/+ and db/db-/- mice contracted with PE, (10^{-5} M). Impaired ACh-induced relaxation was observed in CC from db/db-/- compared to lean db/+ mice (panel B). Enhancement of responses to ACh-induced relaxation was observed in the presence of BAY 41-2272 in CC from db/+ (panel C) and obese, diabetic db/db-/- mice (panel D). Data represent means ± “S.E.M.” of 6 experiments. *P < 0.05; **P < 0.01 “vs.” to db/+.

Figure 2. Concentration-response curves to the nitric oxide donor, SNP (10^{-8} to 10^{-5} M), in CC from db/+ and db/db-/- mice contracted with PE (10^{-5} M) (panel A). The potency values (pEC_{50}) observed for SNP were markedly increased by pre-treatment with 10^{-8}, 3x10^{-8} and 10^{-7} M BAY 41-2272 in CC from db/+ and db/db-/- mice (panel B). Data represent means ± “S.E.M.” of 4 experiments. *P < 0.05 and **P < 0.01, compared with the respective CC control.

Figure 3. Electrical field stimulation (EFS)-induced nitrergic relaxation (1-32 Hz) in the CC from db/+ and diabetic/obese db/db-/- mice (panel A). The magnitude (panel B) and duration (panel C) of EFS-induced relaxations were increased in the presence of 10^{-8}, 3x10^{-8} and 10^{-7} M BAY 41-2272. Representative original traces of 32 Hz in CC from db/+ and db/db-/- mice in the absence or presence of 10^{-8}, 3x10^{-8} and 10^{-7} M BAY 41-2272 are shown in panel D. Data represent means ± “S.E.M.” of 5 experiments. *P < 0.05; **P < 0.01, compared with the respective CC control.
Figure 4. Cyclic GMP contents in corpus cavernosum (CC) from db/+ and db/db⁻/⁻ mice. Tissues previously contracted with PE (10⁻⁵ M) were stimulated for 10 min with: ACh (10⁻⁶ M), BAY 41-2272 (10⁻⁷ M), or their combination (Figure 4A); EFS (4 Hz, 10 seconds), BAY 41-2272 (10⁻⁷ M), or their combination (Figure 4B); SNP (10⁻⁶ M), BAY 41-2272 (10⁻⁷ M) or their combination (Figure 4C). Difference in cGMP levels were observed between strains (db/+ “vs.” db/db⁻/⁻) only in tissue stimulated by EFS (Figure 4B). In CC treated only with BAY the cGMP levels were increased in both strains (Figure 4A, 4B and 4C). Data represent means ± S.E.M. of 5 mice per group. *P < 0.05; **P < 0.01, compared with the respective baseline values (CTL). #P < 0.001 compared with the sum of BAY 41-2271 (10⁻⁷M) and EFS (4Hz), BAY 41-2271 (10⁻⁷M) and SNP (10⁻⁶ M), or both drugs alone, ΔP < 0.05, compared with lean (db/+ in the same experimental condition ( ANOVA one-way followed by Bonferroni post-hoc test).

Figure 5. BAY 41-2272 prevents increased expression of NADPH oxidase subunits in diabetic (db/db⁻/⁻) mice. Enhanced expression of NADPH oxidase subunits, gp91phox (panel A), p22phox (panel B), and p47phox (panel C) was observed in obese, diabetic db/db⁻/⁻ compared to lean db/+ mice. In CC tissues from db/db⁻/⁻ mice, incubation with 10⁻⁸ M and 10⁻⁷ M BAY 41-2272 significantly reduced protein levels of gp91phox (panel D), p22phox (panel E), and p47phox (panel F). Each panel illustrates representative Western Blot images and their respective quantitative analyses. Data represent means ± “S.E.M.” of 4 experiments. *P < 0.05 and **P < 0.01, compared with CTL tissue.
Figure 6. Total antioxidant status (TAS) was evaluated and superoxide anion was measured in mice cavernosal tissue. TAS in plasma and urine samples was markedly decreased in obese, diabetic db/db-/- compared to lean db/+ mice (panel A). Higher levels of superoxide anion were observed in obese, diabetic db/db-/- compared to lean db/+ mice. Incubation with BAY 41-2272 significantly diminished superoxide anion production in CC from db/db-/- (panel B). Data represent means ± “S.E.M.” of 5 experiments. *P < 0.05 and **P < 0.01, “vs.” db/+.
**Table 1- Body weight, glucose and lipid profile (triglycerides, total cholesterol and HDL-cholesterol) of db/+ and db/db-/- mice.** *P<0.05 “vs.” db/+ (t-test). Data represent means ± “S.E.M” for N=6 in each group.

<table>
<thead>
<tr>
<th></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<0.01 compared to the db/+ group.
Figure 1

A

Relaxation (%)

-8 -7 -6 -5

Log [BAY 41-2272] (M)

- db/+ - db/db-/-

B

Relaxation (%)

-8 -7 -6 -5

Log [ACh] (M)

- lean db/+ - db/db-/-

C

Relaxation (%)

-8 -7 -6 -5

Log [ACh] (M)

- CTL - BAY (10^-8 M) - BAY (3x10^-8 M) - BAY (10^-7 M)

- db/+ - db/db-/-

D

Relaxation (%)

-8 -7 -6 -5

Log [ACh] (M)

- CTL - BAY (10^-8 M) - BAY (3x10^-8 M) - BAY (10^-7 M)

- * - **
Figure 2

A

Relaxation (%)

\[ \text{Log [SNP] (M)} \]

pEC\textsubscript{50}:

- db/+ (6.65 ± 0.06)
- db/db \(-/-\) (6.49 ± 0.04)

B

\text{pEC}_{50} \text{ values}

-8 -7.5 -7

-8 -7.5 -7

\text{db/+} \hspace{1cm} \text{db/db \(-/-\)}

 CTL

 BAY

**

*
**Figure 5**

A. 

![Image of gel analysis for gp91phox](image)

B. 

![Image of gel analysis for p22phox](image)

C. 

![Image of gel analysis for p47phox](image)

D. 

![Image of gel analysis for gp91phox](image)

E. 

![Image of gel analysis for p22phox](image)

F. 

![Image of gel analysis for p47phox](image)
**Figure 6**

**A**

![Bar graph showing TAS (mM) in plasma and urine for db/+ and db/db -/- genotypes. The graph indicates a significant difference between genotypes in plasma and urine samples.](image)

**B**

![Bar graph showing nmol O₂/mg protein/h in vehicle and BAY 41-2272 treatment groups for db/+ and db/db -/- genotypes. The graph shows a significant difference in the BAY 41-2272 treatment group.](image)