Soluble Guanylyl Cyclase (sGC) Degradation and Impairment of Nitric Oxide-Mediated Responses in Urethra from Obese Mice: Reversal by the sGC Activator BAY 60-2770

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ABBREVIATIONS:
BAY 41-2272, (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine); BAY 60-2770, acid 4-(((4-carboxybutyl) [2-(5-fluoro-2-[[4-(trifluoromethyl) biphenyl-4-yl] methoxy] phenyl) ethyl] amino) methyl) benzoic; 8-Br-cGMP, 8-bromo-cyclic GMP; cGMP, cyclic guanosine monophosphate; DHE, hydroethidine; GTN, glyceryl trinitrate; LUTS, lower urinary tract symptoms; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; OAB, overactive bladder; ODQ, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one; ROS, reactive-oxygen species; sGC, soluble guanylate cyclase; SNOG, S-nitrosoglutathione; USM, urethral smooth muscle.

Section: Gastrointestinal, Hepatic, Pulmonary, and Renal
ABSTRACT

Obesity has emerged as a major contributing risk factor for overactive bladder (OAB), but no study examined the urethral smooth muscle (USM) dysfunction as a predisposing factor to obesity-induced OAB. This study aimed to investigate the USM relaxant machinery in obese mice, and whether soluble guanylyl cyclase (sGC) activation with BAY 60-2770 rescues the urethral reactivity through improvement of sGC-cGMP signaling. Male C57BL/6 mice were fed for 12 weeks with high-fat diet to induce obesity. Separate groups of animals were treated with BAY 60-2770 (1 mg/kg/day, 2 weeks). Functional assays and measurements of cGMP, reactive-oxygen species (ROS) and sGC protein expression in USM were determined. USM relaxations induced by NO (acidified sodium nitrite), NO-donors (S-nitrosoglutathione and glyceryl trinitrate) and BAY 41-2272 (sGC stimulator) were markedly reduced in obese compared with lean mice. As opposed, USM relaxations induced by BAY 60-2770 (sGC activator) were 43% greater in obese mice ($P<0.05$), which was accompanied by increases in cGMP levels. Oxidation of sGC with ODQ (10 μM) potentiated BAY 60-2770-induced USM responses in lean group. Long-term oral BAY 60-2770 administration fully prevented the impairment of USM relaxations in obese mice. Reactive-oxygen species (ROS) production was enhanced, whereas protein expression of β1 sGC subunit was reduced in USM from obese mice, both of which were restored by BAY 60-2770 treatment. In conclusion, impaired USM relaxation in obese mice is associated with ROS generation and downregulation of sGC-cGMP signalling. Prevention of sGC degradation by BAY 60-2770 ameliorates the impairment of urethral relaxations in obese mice.
Introduction

The metabolic syndrome or syndrome X is a term that describes a group of independent risk factors (central obesity, insulin resistance, dyslipidemia and high blood pressure) for the development of type 2 diabetes and cardiovascular diseases, which is estimated to affect 47 million American residents (Ford et al., 2002; Hutcheson and Rocic, 2012). Recent studies have implicated metabolic syndrome/obesity as a major contributing factor for lower urinary tract symptoms (LUTS) that is positively correlated with overactive bladder (OAB) (Steers, 2009; Richter et al., 2010). The prevalence of OAB and metabolic syndrome in the U.S. adult population is 16% and 26%, respectively (Irwin et al., 2011). Animal models have provided evidence to confirm a relationship between metabolic syndrome/hyperlipidemia and OAB symptoms (Rahman et al. 2007; Nobe et al., 2008; Lee et al., 2011; Gasbarro et al., 2010). Obesity-associated insulin resistance has been shown to play an important role in OAB pathophysiology in mice (Leiria et al., 2012).

The lower urinary tract consists of the urinary bladder and urethra. The urethra contributes to urinary continence by relaxing during the voiding phase and contracting during the urine storage phase (Michel and Vrydag, 2006). Urethral smooth muscle is richly innervated by sympathetic fibers resulting in the release of noradrenaline that acts on post-junctional α1-adrenoceptors leading to urethral contractions. This is the main excitatory pathway responsible for the urethral smooth muscle contraction that maintains continence (Michel and Vrydag, 2006). Urethral smooth muscle tone is also under the control of non-adrenergic non-cholinergic (NANC) inhibitory innervation (Fraser and Chancellor, 2003). Nitric oxide (NO) acts as the main neurotransmitter involved in mediating the relaxant response of urethral smooth muscle (Persson and
Andersson, 1992; Dokita et al., 1994), thus importantly contributing to the maintenance of the urinary continence (Bennett et al., 1995). In response to NO stimulation, soluble guanylyl cyclase (sGC) catalyzes the conversion of GTP to cGMP, which leads to relaxation of different types of smooth muscle (Friebe and Koesling, 2003). Direct sGC stimulation causes cGMP-dependent urethral relaxations (Costa et al., 2001) in a synergistic fashion with NO (Toque et al., 2008).

Activators of sGC like BAY 60-2770 and BAY 58-2667 acts by NO- and heme-independent mechanisms (Schmidt et al., 2009). These compounds are reported to protect sGC from heme oxidation in smooth muscle tissues (Stasch et al., 2006; Meurer et al., 2009; Jones et al., 2010; Lasker et al., 2013), ameliorating OAB in obese mice (Leiria et al., 2013). Additionally, long-term sGC stimulation counteracts the voiding dysfunction in chronically NO-deficient rats (Mónica et al., 2011). Although urethra is a critical structure in the lower urinary tract that contributes to urinary incontinence (Torimoto et al., 2004), no study to date has examined the impairment of urethral smooth muscle relaxations as a predisposing factor to obesity-induced OAB. Since activation of the NO/sGC/cGMP signaling pathway may cause smooth muscle relaxations at the level of urethra thus reducing the bladder outlet obstruction, targeting this pathway may be of benefit to treat OAB. In the present study we aimed to investigate the impaired NO/sGC/cGMP signaling pathway in urethral smooth muscle of high-fat fed mice, and whether the sGC activator BAY 60-2770 rescues the urethral function through the improvement of sGC activity and cGMP production.
Materials and Methods

Animals. All animal procedures and the experimental protocols were according to the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA) and approved by the institutional Committee for Ethics in Animal Research/State University of Campinas (CEEA-UNICAMP). Four-week-old male C57BL6/J mice were provided by the Central Animal House Services of State University of Campinas (UNICAMP). The animals were housed two per cage on a 12 h light–dark cycle, and fed for 12 weeks with either a standard chow diet (carbohydrate: 70%; protein: 20%; fat: 10%) or a high fat diet that induces obesity (carbohydrate: 29%; protein: 16%; fat: 55%) (Leiria et al., 2012).

In vitro Functional Assays and Concentration-Response Curves. Mice were sacrificed in CO₂ chamber. Urethra was removed and cut into rings (1 to 1.5 mm in length). Urethral smooth muscle rings were mounted in 5 ml organ baths containing Krebs-Henseleit solution (mM: 117 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃ and 11 glucose) continuously bubbling with a mixture of 95% O₂ e 5% CO₂ at pH 7.4, 37°C. Changes in isometric force were recorded using a Power Lab v.7.2 system (ADInstruments, Sydney, Australia). The resting tension was adjusted to 2 mN at the beginning of the experiments. The equilibration period was 45 min and the bathing medium was changed every 15 min until the start of the experiment. Urethral rings were pre-contracted with the α₁-adrenoceptor agonist phenylephrine (10 μM). Once the contraction had reached plateau, cumulative concentration–response curves to the following relaxant agents were obtained using one-half log unit: NO (added as acidified nitrite solution; 0.001-300 μM), S-nitrosoglutathione (SNOG; 0.001-100 μM), glycercyl trinitrate (GTN; 0.001-100 μM), BAY 41-2272 (sGC stimulator; 0.0001-30
μM), BAY 60-2770 (sGC activator; 0.0001-30 μM), tadalafil (phosphodiesterase-5 inhibitor; 0.0001-10 μM) or 8-Br-cGMP (cell-permeable cGMP analogue; 0.003-100 μM). One concentration–response curve to each relaxing agent was obtained in each urethral preparation. For BAY 41-2272 and BAY 60-2770, control experiments were carried out in urethral rings in the presence of 0.22 - 0.32% DMSO (vehicle used to dissolve these drugs). In separate urethral preparations, concentration-response curves were repeated in the presence of the sGC inhibitor ODQ (10 μM), pre-incubated for 30 min. Nonlinear regression analysis to determine the pEC$_{50}$ was carried out using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/([1 + (10^c/10^x)n] + F)$, where $E$ is the maximum response produced by agonists; $c$ is the logarithm of the EC$_{50}$, the concentration of drug that produces a half-maximal response; $x$ is the logarithm of the concentration of the drug; the exponential term, $n$, is a curve-fitting parameter that defines the slope of the concentration–response line, and $F$ is the response observed in the absence of added drug. Relaxing responses were calculated as percentages of the maximal changes from the steady-state contraction produced by phenylephrine (10 μM) in each tissue. Data are shown as the percentage of relaxation of $n$ experiments, expressed as the mean ± S.E.M. EC$_{50}$ values are presented as the negative logarithm (pEC50) and calculated by a fitting concentration–response relationship to a sigmoidal model of the form log-concentrations vs response using GraphPad Software.

**Determination of cGMP Levels.** After animals were sacrificed in CO$_2$ chamber, urethras were immediately excised and equilibrated for 45 min in oxygenated Krebs solution. Tissues were then stimulated for 10 min with either GTN (10 μM) or BAY 60-
2770 (10 μM) alone and in the presence of the sGC inhibitor 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; 10 μM), and immediately frozen in liquid nitrogen. Tissues were pulverized and subsequently processed for cGMP measurement using ELISA immunoassay kit according to the manufacturer’s protocol (Cayman Chemical Cyclic GMP EIA kit, Ann Arbor, MI, USA). The assays were performed in duplicates, and quantification of protein by Lowry method was used to normalize the data as pmol/μg protein. A pool of 5 urethras was used to constitute each experimental n.

**Measurement of Reactive-Oxygen Species (ROS).** The oxidative fluorescent dye hydroethidine (DHE; Invitrogen, Grand Island, NY, USA) was used to evaluate in situ ROS generation. The urethra was embedded in a freezing medium and transverse sections (12 μm) of frozen tissue obtained on a cryostat, collected on glass slides and equilibrated for 10 min in Hanks solution (in mM: 1.6 CaCl₂; 1.0 MgSO₄; 145.0 NaCl; 5.0 KCl; 0.5 NaH₂PO₄; 10.0 dextrose; 10.0 HEPES; pH 7.4) at 37°C. Fresh Hanks solution containing hydroethidine (2 μM) was topically applied to each tissue section and the slices were incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained with microscope Eclipse 80i, Nikon, Japan) equipped for epifluorescence (excitation at 488 nm; emission at 610 nm) and camera (DS-U3, Kilon, Japan). Fluorescence was detected with a 585 nm long-pass filter. The number of nuclei labeled with ethidium bromide (EB-positive nuclei) along urethra wall was automatically counted using Image J Software (National Institute of Health, Bethesda-MD, USA) and expressed as labeled nuclei/mm².
Western Blotting for $\beta_1$-subunit in urethral tissues. Urethral tissues were isolated, washed in Krebs-Henseleit solution and homogenized in SDS lysis buffer with Ultrasonic processor (model VCX130; Sonic & Materials Inc., Newtown, CT, USA) and centrifuged (12,000 $\times$ g, 4°C, 30 min). Protein concentrations of the supernatants were determined by the Bradford assay, and equal amount of protein from each sample (70 $\mu$g) was treated with Laemmli buffer containing dithiothreitol (100 mM). Samples were heated in a boiling water bath for 10 min and resolved by SDS-PAGE. The proteins were separated by 12% polyacrylamide gels and then electrotransfered to nitrocellulose membrane, performed for 1 h at 15 V (constant) in a semi-dry device (Bio-Rad, Hercules, CA, USA). Nonspecific protein binding to nitrocellulose was reduced by pre-incubating the membrane overnight at 4°C in blocking buffer (0.5% non-fat dried milk, 10 mM Tris, 100 mM NaCl, and 0.02% Tween 20). Detection using specific antibodies, HRP-conjugated secondary antibodies, and luminol solution was performed. Anti-sGC $\beta_1$-subunit and anti $\alpha$-actin antibodies were obtained from Novus Biologicals (Oakville, ON, CA). Densitometry was performed using the Scion Image Software (Scion Corporation, Frederick, MD).

Statistical Analysis. Data are expressed as mean ± S.E.M. of $n$ experiments. The program Instat (GraphPad Software) was used for statistical analysis. Statistical comparisons were made using one-way analysis of variance (ANOVA), and Tukey method was chosen as a post-test. Student’s unpaired t-test was also used when appropriate. $P < 0.05$ was accepted as significant.
Results

Impairment of NaNO₂-, SNOG- and GTN-induced urethral smooth muscle relaxations in obese mice. Phenylephrine (10 μM) produced sub-maximal contraction in the urethral smooth muscle preparations that did not differ between lean and obese mice (2.00 ± 0.27 and 2.10 ± 0.36 mN in lean and obese mice, respectively).

Cumulative addition of NaNO₂ (0.001-300 μM), SNOG (0.001-100 μM) and GTN (0.001-100 μM) produced concentration-dependent urethral relaxations in lean group (Fig. 1, A, B and C). Prior treatment of urethra preparations from lean group with the sGC inhibitor ODQ (10 μM, 30 min) markedly reduced the NaNO₂-, SNOG- and GTN-induced urethral relaxations, as demonstrated by the Eₘₐₓ values (Table 1).

In obese group urethral relaxations induced by NaNO₂, SNOG and GTN were markedly reduced as compared with lean groups (Fig. 1, A, B and C; Table 1). Prior treatment with ODQ further reduced NaNO₂-induced relaxations in the obese group (P < 0.05). No significant differences for the potency (pEC₅₀) values for NaNO₂, SNOG and GTN were found between lean and obese groups, treated or not with ODQ, except for obese treated with ODQ where a higher pEC₅₀ value was found (Table 1).

Differential relaxant effects of sGC stimulator (BAY 41-2272) and activator (BAY 60-2770) in urethral smooth muscle of obese and lean mice. Similarly to the NO donors, addition of the sGC stimulator BAY 41-2272 (0.0001-30 μM) promoted concentration-dependent urethral relaxations in lean group that were significantly reduced by pre-incubation with ODQ (10 μM, 30 min). In obese mice, the relaxant responses to BAY 41-2272 were reduced compared with lean group, and pre-incubation with ODQ further decreased the urethral relaxations (Fig. 2A; Table 2). No significant
differences for the pEC$_{50}$ values for BAY 41-2272 were found between the lean and obese groups (Table 2).

Cumulative addition of the sGC activator BAY 60-2770 (0.0001-30 μM) to the urethral smooth muscle preparations produced a different pattern of response (Fig. 2B; Table 2). BAY 60-2770 promoted concentration-dependent urethral relaxations in lean group that were rather potentiated by ODQ. In addition, E$_{\text{max}}$ and pEC$_{50}$ values to BAY 60-2770 were 43% greater in obese compared with lean mice ($P < 0.05$).

**Urethral relaxations to tadalafil and 8-Br-cGMP.** Cumulative addition of the PDE5 inhibitor tadalafil (0.0001-10 μM) or the permeable cGMP analogue 8-Br-cGMP (0.003-100 μM) produced concentration-dependent urethral relaxations in lean and obese groups. However, no statistical differences between both groups were found, as observed at the level of E$_{\text{max}}$ values and pEC$_{50}$ values (Table 3).

**Levels of cGMP in urethral tissues.** Incubation of urethral tissues from lean mice with GTN (10 μM, 10 min) elevated by 2.1-fold ($P < 0.05$) the cGMP levels above basal levels (0.78 ± 0.008 and 1.67 ± 0.13 pmol/μg for basal and stimulated, respectively; $n = 3$). In obese group, however, GTN (10 μM, 10 min) failed to elevate significantly the cGMP levels (0.69 ± 0.09 and 0.93 ± 0.02 pmol/μg for basal and stimulated, respectively).

Incubation of urethral tissues with BAY 60-2770 (10 μM; $n = 3-4$) produced a markedly greater elevation in cGMP levels in obese compared with lean group ($P < 0.05$; Fig. 3). Incubation of urethral tissues with ODQ (10 μM) prior to the stimulation with BAY 60-2770 further elevated the cGMP levels in lean, but not in obese group.
The vehicle DMSO (0.22%) alone had no effect on intracellular levels of cGMP in any condition.

Chronic treatment with BAY 60-2770 on urethral relaxations, sGC expression and reactive-oxygen species (ROS) production. Given that sGC activators reactivate the heme-oxidized enzyme in vascular diseases and platelets (Stasch et al., 2006) and ameliorate obesity-associated OAB (Leiria et al., 2013), we next moved on to examine the effects of long-term BAY 60-2770 treatment on the impairment of urethral relaxations in obese mice. To achieve this, lean and obese mice were orally treated with BAY 60-2770 (1 mg/kg/day, given as daily gavage from the 10th to 12th week) or its vehicle (Transcutol® : Cremophor® : Water, 1:2:7, v/v/v), according to our previous experience (Leiria et al., 2013). Thereafter, concentration-response curves to NaNO2, protein expression of β1 subunit of sGC and reactive-oxygen species (ROS) levels were evaluated in urethral tissues in all groups.

Long-term treatment with BAY 60-2770 did not interfere on the amplitude of contractions induced by phenylephrine in both lean and obese mice (2.14 ± 0.45 and 2.05 ± 0.51 mN, respectively). Long-term treatment with BAY 60-2770 fully prevented the impairment of NaNO2 (0.001-300 μM)-induced urethral relaxations in obese mice without affecting the responses in lean group (n = 6, P < 0.01; Fig. 4).

As demonstrated in figure 5, protein expression of β1 subunit of sGC were 37% lower in the urethral tissues from obese group in comparison with lean mice (P <0.05, n = 7-10). Oral treatment with BAY 60-2770 fully restored the protein levels of β1 subunits to those of lean group.

Reactive-oxygen species in urethra was measured by fluorescent dye DHE in fresh frozen sections of urethra from lean and obese mice treated or not with BAY60-
2770. Fluorescence intensity was 113% higher in the urethral smooth muscle of obese compared with lean group ($P < 0.05$, $n = 5$). Treatment with BAY 60-2770 restored the ROS levels in obese mice to control levels ($P < 0.05$, Fig. 6).
Discussion

The present study shows an impairment of NO-cGMP-dependent urethral smooth muscle relaxation in obese mice that is associated with enhanced ROS production and decreased protein levels of β1 subunit of sGC in the urethral tissues. Moreover, prolonged administration of the sGC activator BAY 60-2770 reversed the functional and molecular alterations observed in urethra of obese mice.

Obesity is an important public health problem greatly elevating the risks of urological complications such as OAB and urinary incontinence. Alterations in both urodynamic profile and bladder reactivity in vitro have been described in different animal models of obesity (Rahman et al., 2007; Lee et al., 2011; Gasbarro et al., 2010; Leiria et al., 2012). However, no study has evaluated the potential implication of impairment of urethral smooth muscle relaxations contributing to the overall obesity-related micturition problems. Using a model of obesity-associated voiding dysfunction, we initially designed experiments to evaluate the in vitro urethral smooth muscle reactivity to agents that interfere at different levels with the NO-sGC-cGMP-PDE5 signaling pathway. These agents included NO donors (acidified NaNO2, SNOG and GTN), sGC stimulator (BAY 41-2272), sGC activator (BAY 60-2770), PDE5 inhibitor (tadalafil) and a permeable cGMP analogue (8-Br-cGMP). Biological effects of nitrates (GTN) and nitrosothiols (SNOG) are attributable to NO formation via enzymatic or non-enzymatic bioactivation. Acidification of NaNO2 yields nitrous acid that spontaneously decomposes to NO and other nitrogen oxides (Wang et al., 2002; Lundberg et al., 2005). In control group, NaNO2, SNOG and GTN produced concentration-dependently urethral relaxations that were markedly reduced by oxidation of heme moiety (Fe3+) of sGC with ODQ, indicating a major for cGMP in mediating
these relaxing responses. Moreover, urethral relaxations to NaNO₂, SNOG and GTN were largely reduced in obese mice in an ODQ-resistant manner, indicating that deficiency of cGMP production accounts for the impairment of urethral smooth muscle relaxations in adiposity conditions. Accordingly, GTN markedly elevated the cGMP levels in urethral tissues of lean but not of obese mice. It is unlikely that defects for urethral relaxations in obese mice rely on signal-transduction components downstream of cGMP generation since tadalafil and 8-Br-cGMP-induced responses remained unchanged between groups. We therefore hypothesized that impairment of urethral smooth muscle relaxations in obesity takes place at the level of sGC.

Soluble guanylyl cyclase is a heterodimeric heme-containing enzyme, consisting of α- and heme-containing β-subunit that converts guanosine triphosphate (GTP) to cGMP. Stimulators and activators of sGC have been developed over the past decade (Stasch et al., 2001; 2002). They comprehend a part of two novel groups of small molecule compounds that increase the enzymatic activity of sGC. The effectiveness of these compounds differs depending on the oxidation state of sGC enzyme. Similarly to endogenous ligand NO, sGC stimulators like BAY 41-2272 increase sGC activity only when the heme iron is in its reduced state (Fe²⁺). On the other hand, the sGC activators like BAY 58-2667 and BAY 60-2770 preferentially induces sGC activation when the heme iron is in its oxidized state (Fe³⁺ instead of Fe²⁺) or even when the heme group is missing (Stasch et al., 2002). In our study, BAY 41-2272 produced ODQ-sensitive urethral relaxations in lean mice that were largely reduced in obese group. Previous studies have reported the inhibitory actions of ODQ on BAY 41-2272-induced aorta relaxations (Priviero et al., 2005; Teixeira et al., 2006). As opposed to BAY 41-2272, incubation with ODQ rather potentiated the urethral relaxations induced by the sGC activator BAY 60-2770 in lean mice. BAY 60-2770-induced relaxations were also
greater in obese compared with lean mice. The enhanced functional responses by BAY 60-2770 are consistent with the higher cGMP levels in urethral tissues of obese mice (or lean in the presence of ODQ). Two mechanisms have been proposed to explain the mode of action of sGC activators: 1) these compounds inducing and accelerating heme loss from ferric sGC or 2) they occupying the heme site in conditions where sGC is oxidized, avoiding degradation of α- and β sGC subunits. BAY 58-2667-induced responses were greater in aorta from spontaneously hypertensive rats (SHR) and mesocolon arteries from type 2 diabetic patients (Stasch et al., 2006). Additionally, the positive interaction of ODQ with sGC activator on cGMP concentrations has been previously reported in porcine endothelial cells (Stasch et al., 2006) and corpus cavernosum (Lasker et al., 2013). Therefore, it is likely that heme group of sGC is oxidized in urethra smooth muscle from obese mice.

A chronic state of oxidative stress is a hallmark of cardiovascular and endocrine-metabolic diseases (Paravicini et al, 2008). The oxidative stress appears to shift the balance to the NO-insensitive oxidized state leading to downregulation of sGC, which may take place through S-nitrosylation (Sayed et al., 2007; Mayer et al., 2009). For instance, sGC protein expression is decreased in vascular smooth muscle of hypertensive rats (Ruetten et al., 1999; Klöss et al., 2000) and hypercholesterolemic rabbits (Melichar et al., 2004), possibly as a consequence of chronic oxidative stress (Priviero et al., 2009; Kagota et al., 2013). Reduction of protein levels of α1 and/or β1 subunits of sGC by oxidation of heme moiety (Fe³⁺) was also reported in cGMP reporter cell line (Hoffmann et al., 2009) as well as in cultured vascular smooth muscle cells from obese rats (Russo et al., 2008).

Given that sGC activators are able to reactivate heme-oxidized sGC (Stasch et al., 2006) and ameliorate obesity-associated overactive bladder (Leiria et al., 2013), we...
next investigated the effects of prolonged BAY 60-2770 administration on the functional and molecular alterations of urethra smooth muscle and its association with obesity. Two-week oral treatment with BAY 60-2770 fully restored the impaired NO-mediated urethral relaxations in obese mice, without affecting the responses in lean group. Additionally, the expression of the β1 subunit of sGC was reduced whereas ROS production was enhanced in the urethral tissues from obese in comparison with lean mice, an effect restored by oral treatment with BAY 60-2770. Therefore, local ROS generation is likely to account for the heme-oxidation of β1 subunit of sGC in the urethra of obese mice. The effect of BAY 60-2770 on sGC protein levels is hypothesized to be in virtue of sGC subunits stabilization upon BAY 60-2770 binding to its heme pocket. In porcine endothelial and smooth muscle cells, oxidation of sGC with ODQ decreased sGC protein levels, indicating an ubiquitin-dependent protein degradation rather than inhibition of protein synthesis. Besides, the sGC activator BAY 58-2667 prevented the decrease on sGC protein levels induced by heme oxidants (Stasch et al., 2006; Hoffmann et al., 2009). However, the role of redox state of sGC in regulating stability and protein levels remains unclear. Of interest, a recent study showed that heme domain of sGC contains an S-nitrosylation site (β1 C122) involved in enzyme desensitization, and BAY 60-2770 facilitates the displacement of heme from ferric sGC or alternatively binds to the vacant heme pocket of apo sGC (Kumar et al., 2013). Adiposity markedly increases ROS levels in bladder (Leiria et al., 2013) and urethral tissues (present study). Two-week therapy with BAY 60-2770 did not significantly affect ROS levels in bladder, but rather normalized ROS levels in urethra. The density of positive nerves for nitric oxide synthase is greater in bladder neck and proximal urethra than bladder, and that the NO-cGMP signaling pathway is more active in urethra (Uckert and Kuczyk, 2011). Interestingly, a previous study showed that
urethra is more sensitive to ischemic injury than the bladder (Bratslavsky et al., 2001). It is possible therefore that BAY 60-2770 causes a more efficient vasodilatation in the urethral vascular bed, ameliorating the blood perfusion, thus accelerating the clearance of ROS levels in this tissue.

In summary, our results show that the obese mice display urethral dysfunction associated with sGC oxidation and impairment of sGC-dependent urethral relaxation. Two-week therapy with the sGC activator BAY 60-2770 increases the expression of β1-subunits of sGC in the urethral tissues and reduces ROS formation resulting in amelioration of urethra dysfunction in high-fat fed obese mice. The present work highlights the possibility of targeting urethral smooth muscle to treat oxidative stress-related bladder dysfunction.
Authorship Contributions

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*Performed data analysis:* E.C. Alexandre, L.O. Leiria, E. Antunes

*Wrote or contributed to the writing of the manuscript:* E.C. Alexandre, L.O. Leiria, E. Antunes
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incontinence symptoms, severity, urodynamic characteristics and quality of life.

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Figure legends

**Fig. 1.** Concentration-response curves to acidified nitrite solution (NaNO₂; A and B), S-nitrosoglutathione (SNOG; C and D) and glyceryl trinitrate (GTN; E and F) in urethra smooth muscle from lean and obese mice in the absence and the presence of 1H-[1,2,4] oxadiazolo [4,3,a]quinoxalin-1-one (ODQ; 10 μM). Relaxations were calculated relative to the maximal changes from the contraction produced by phenylephrine (10 μM) in each urethral ring, which was taken as 100%. Data are presented as mean ± S.E.M. (n = 4-6). *P < 0.05, **P < 0.01 lean vs obese (ANOVA followed by a Tukey test).

**Fig. 2.** Concentration-response curves to BAY 41-2272 (A and B) and BAY 60-2770 (C and D) in urethra smooth muscle from lean and obese mice in the absence and the presence of 1H-[1,2,4] oxadiazolo [4,3,a]quinoxalin-1-one (ODQ; 10 μM). Relaxations were calculated relative to the maximal changes from the contraction produced by phenylephrine (10 μM) in each urethral ring, which was taken as 100%. Data are presented as mean ± S.E.M. (n = 4-8). *P < 0.05, **P < 0.01 lean vs obese (ANOVA followed by a Tukey test).

**Fig. 3.** Cyclic GMP content in urethral tissues from lean and obese mice. Tissues were stimulated with BAY 60-2770 (10 μM) alone and in the presence of 1H-[1,2,4] oxadiazolo [4,3,a]quinoxalin-1-one (ODQ, 10 μM). Data are presented as mean ± S.E.M. of 3-4 animals.*P < 0.05 compared with respective non-stimulated lean group (Basal). † P < 0.05 compared with lean in absence of ODQ.
**Fig. 4.** Concentration-response curves to acidified nitrite solution (NaNO₂; A and B) in urethra smooth muscle from lean and obese mice, treated chronically or not with BAY 60-2770 (1 mg/kg, 2 weeks). (C) Maximal responses (E₂₅₆₆) values for all groups. Relaxations were calculated relative to the maximal changes from the contraction produced by phenylephrine (10 μM) in each urethral ring, which was taken as 100%. Data are presented as mean ± S.E.M. (n = 6). *P < 0.05 lean vs obese (ANOVA followed by a Tukey test).

**Fig. 5.** (A) Representative images of Western blotting for β₁ subunit of soluble guanylyl cyclase (sGC) in homogenates of urethra from lean and obese mice, treated chronically or not with BAY 60-2770 (1 mg/kg, 2 weeks). (B) Protein values for β₁ subunit of sGC / α-actin in all groups. Data represent the mean ± S.E.M. of 7 to 10 animals each group. *P < 0.05 in comparison with lean + vehicle.

**Fig. 6.** Reactive-oxygen species (ROS) levels through dye hydroethidine (DHE)-induced fluorescence in urethral tissues of lean from lean and obese mice, treated chronically or not with BAY 60-2770 (1 mg/kg, 2 weeks). Data are presented as mean ± S.E.M. (n = 5 each group). *P < 0.05 in comparison with lean + vehicle. Magnification: 200X.
Table 1

Maximal responses (E_max) and potency (pEC_{50}) values derived from concentration–response curves to acidified nitrite solution (NaNO_2; 0.001-300 μM), S-nitrosoglutathione (SNOG; 0.001-100 μM) and glycercyl trinitrate (GTN; 0.001-100 μM) in urethra smooth muscle from lean and obese mice in the presence and absence of 1H-[1,2,4] oxadiazolo [4,3,-a]quinoxalin-1-one (ODQ; 10 μM)

<table>
<thead>
<tr>
<th>Groups</th>
<th>NaNO_2</th>
<th>SNOG</th>
<th>GTN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E_max (%)</td>
<td>pEC_{50}</td>
<td>E_max (%)</td>
</tr>
<tr>
<td>Lean</td>
<td>51.2 ± 1.9</td>
<td>6.58 ± 0.07</td>
<td>66.3 ± 7.3</td>
</tr>
<tr>
<td>Obese</td>
<td>36.5 ± 0.8**</td>
<td>6.42 ± 0.04</td>
<td>36.3 ± 4.2**</td>
</tr>
<tr>
<td>Lean + ODQ</td>
<td>18.2 ± 5.2**</td>
<td>6.65 ± 0.22</td>
<td>28.1 ± 0.8**</td>
</tr>
<tr>
<td>Obese + ODQ</td>
<td>20.3 ± 7.4*</td>
<td>6.77 ± 0.35</td>
<td>27.4 ± 2.2</td>
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</tbody>
</table>

Data are presented as mean ± SEM (n = 4-6). Values of E_max are represented as %. Data represent the means ± SEM (n = 4-6). *P < 0.05, **P < 0.01 compared with respective untreated lean group; and #P < 0.05 compared with obese group (ANOVA followed by a Tukey test).
Table 2

Maximal responses (E$_{\text{max}}$) and potency (pEC$_{50}$) values derived from concentration–response curves to BAY 41-2272 (0.0001-30 μM) and BAY 60-2770 (0.0001-30 μM) in urethra smooth muscle from lean and obese mice in the presence and absence of 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ; 10 μM).

<table>
<thead>
<tr>
<th>Groups</th>
<th>BAY 41-2272</th>
<th>BAY 60-2770</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E$_{\text{max}}$ (%)</td>
<td>pEC$_{50}$</td>
</tr>
<tr>
<td>Lean</td>
<td>75.9 ± 2.3</td>
<td>6.65 ± 0.05</td>
</tr>
<tr>
<td>Obese</td>
<td>60.9 ± 0.5*</td>
<td>6.46 ± 0.06</td>
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<tr>
<td>Lean + ODQ</td>
<td>54.4 ± 3.9**</td>
<td>6.91 ± 0.07</td>
</tr>
<tr>
<td>Obese + ODQ</td>
<td>42.0 ± 3.5#</td>
<td>6.49 ± 0.08</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM ($n$ = 4-8). Values of E$_{\text{max}}$ are represented as %. *$P$ < 0.05, **$P$ < 0.01 compared with respective untreated lean group; and #$P$ < 0.01 compared with obese group (ANOVA followed by a Tukey test).
Table 3

Maximal responses ($E_{max}$) and potency ($pEC_{50}$) values derived from concentration–response curves to tadalafil (0.0001-10 μM) and 8-Br-cGMP (0.003-100 μM) in urethra smooth muscle from lean and obese mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tadalafil</th>
<th>8-Br-cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{max}$ (%)</td>
<td>$pEC_{50}$</td>
</tr>
<tr>
<td>Lean</td>
<td>51.2 ± 7.6</td>
<td>8.68 ± 0.19</td>
</tr>
<tr>
<td>Obese</td>
<td>50.2 ± 4.2</td>
<td>8.62 ± 0.13</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM (n = 5-8). Values of $E_{max}$ are represented as %.
Figure 3

This figure shows the cGMP (pmol/μg of protein) levels for Lean and Obese samples with different treatments. The x-axis represents the treatments, while the y-axis represents the cGMP levels. The treatments include BAY 60-2770 (10 μM) and ODQ (10 μM). The bars represent the mean ± SEM, with statistical significance indicated by asterisks (*) and a dagger (†), respectively.