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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ABSTRACT

Obesity has emerged as a major contributing risk factor for overactive bladder (OAB), but no study examined urethral smooth muscle (USM) dysfunction as a predisposing factor to obesity-induced OAB. This study investigated the USM relaxant machinery in obese mice and whether soluble guanylyl cyclase (sGC) activation with BAY 60-2770 (sGC activator) was capable of improving USM relaxations in obese mice.

Introduction

The metabolic syndrome (or syndrome X) 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 U.S. 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, which 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 urinary storage phase (Michel and Vrydag, 2006). Urethral smooth muscle (USM) is richly innervated by sympathetic fibers, resulting in the release of noradrenaline that acts on postjunctional α1-adrenoceptors and leading to urethral contractions. This is the main excitatory pathway responsible for the USM contraction that maintains continence (Michel and Vrydag, 2006).

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ABBREVIATIONS: ANOVA, analysis of variance; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b] pyridin-3-yl]-pyrimidin-4-ylamine (sGC stimulator); BAY 58-2667, 4-(((4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}amino)methyl)benzoic acid; BAY 60-2770, acid 4-({(4-carboxybutyl)[1-(2-fluoro-2-[[4-(trifluoromethyl) biphenyl-4-yl]methoxy]phenyl]ethyl}amino)methyl)benzoic; DEPE5, phosphodiesterase-5; DHE, hydroethidine; DHE, hydroethidine; DHE, hydroethidine; GTN, glyceryl trinitrate; NO, nitric oxide; ODQ, 1H-[1,2,4] oxadiazolo[4,3-a]quinolinaxin-1-one; PDE5, phosphodiesterase-5; ROS, reactive-oxygen species; SGC, soluble guanylyl cyclase; SNOG, S-nitrosoglutathione; USM, urethral smooth muscle.
had reached a plateau, cumulative concentration–response curves were repeated in the presence of the sGC inhibitor ODQ [1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (10 μM), preincubated for 30 minutes. A nonlinear regression analysis to determine the pEC50 was performed using GraphPad Prism (GraphPad Software, San Diego, CA) with the constraint that F = 0. All concentration–response data were evaluated for a fit to a logistics function in the form: $E = E_{max} \left[1 + \left(10 / 10^\text{clogEC50}\right) + F\right]$, where $E$ is the maximum response produced by agonists; $c$ is the logarithm of the EC50, 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 the percentage of the maximal changes from the steady-state contraction in the presence of added drug. Relaxing responses were calculated as the percentage of the maximal changes from the steady-state contraction in the presence of added drug.

**Measurement of Reactive-Oxygen Species.** The oxidative fluorescent dye hydroethidine (dibydroethidium, DHE; Invitrogen, Grand Island, NY) was used to evaluate in situ reactive-oxygen species (ROS) generation. The urethra was embedded in a freezing medium, and transverse sections (12 μm) of frozen tissue were obtained on a cryostat, collected on glass slides, and equilibrated for 10 minutes 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 DHE (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 minutes. Images were obtained with a microscope (Eclipse 80i; Nikon, Tokyo, Japan) equipped for epifluorescence (excitation at 488 nm; emission at 610 nm) and camera (DS-U3; Nikon). Fluorescence was detected with a 585-nm long-pass filter. The number of nuclei labeled with ethidium bromide (EB-positive nuclei) along the urethral wall was automatically counted using ImageJ Software (National Institutes of Health, Bethesda, MD) and expressed as labeled nuclei per millimeter squared.

**Western Blotting for β1-Subunit in Urethral Tissues.** Urethral tissues were isolated, washed in Krebs-Henseleit solution, and homogenized in SDS lysis buffer with a Ultrasonic processor (model VCX130; Sonic & Materials, Newtown, CT) and centrifuged (12,000g, 4°C, 30 minutes). Protein concentrations of the supernatants were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Western blotting was performed as previously described (19). Blots were incubated with 1:2000 dilutions of antibodies (Abcam, Cambridge, MA) raised against the α1-adrenoceptor (1:1000 dilution) for 1 hour at room temperature, then washed, and incubated for 1 hour at room temperature with the appropriate secondary antibodies (1:5000 dilution). Bands were visualized using chemiluminescence on the ImageJ Software (National Institutes of Health, Bethesda, MD). Blots were then stripped and reprobed for β1- and β3-adrenoceptor (1:1000 dilution) as loading controls. To quantify the relative expression of the α1-adrenoceptor to β1- and β3-adrenoceptor, the integrated optical density of the α1-adrenoceptor and β1- and β3-adrenoceptor bands were determined.
determined by the Bradford assay, and an equal amount of protein from each sample (70 µg) was treated with Laemmli buffer containing dithiothreitol (100 mM). Samples were heated in a boiling water bath for 10 minutes and resolved by SDS-PAGE. The proteins were separated by 12% polyacrylamide gels and then electrotransferred to nitrocellulose membrane, performed for 1 hour at 15 V (constant) in a semidry device (Bio-Rad Laboratories, Hercules, CA). Nonspecific protein binding to nitrocellulose was reduced by preincubating the membrane overnight at 4°C in blocking buffer (0.5% nonfat dried milk, 10 mM Tris, 100 mM NaCl, and 0.02% Tween 20). Detection using specific antibodies, horseradish peroxidase–conjugated secondary antibodies, and luminol solution was performed. Anti-sGC β1-subunit and anti–α-actin antibodies were obtained from Novus Biologicals (Oakville, ON, Canada). 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 the Tukey method was chosen as a posttest. Student's unpaired t test was also used when appropriate. P < 0.05 was considered statistically significant.

**Results**

**Impairment of NaNO2-, SNOG-, and GTN-Induced USM Relaxations in Obese Mice.** Phenylephrine (10 µM) produced submaximal contraction in the USM preparations, which did not differ between the 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 the lean group (Fig. 1, A–C). Prior treatment of the urethra preparations from the lean group with the sGC inhibitor ODQ (10 µM, 30 minutes) markedly reduced the NaNO2-, SNOG-, and GTN-induced urethral relaxations, as demonstrated by the E_max values (Table 1).

In the obese group, urethral relaxations induced by NaNO2, SNOG, and GTN were markedly reduced as compared with the lean group (Fig. 1, A–C; Table 1). Prior treatment with ODQ further reduced the NaNO2-induced relaxations in the obese group (P < 0.05). No statistically significant differences for the potency (pEC50) values for NaNO2, SNOG, and GTN were found between the lean and obese groups, treated or not with ODQ, except for the obese mice 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 USM of Obese and Lean Mice.** Similarly to the NO donors, the addition of the sGC stimulator BAY 41-2272 (0.0001–30 µM) promoted concentration-dependent urethral relaxations in the lean group that were significantly reduced by preincubation with ODQ (10 µM, 30 minutes). In obese mice, the relaxant responses to BAY 41-2272 were reduced compared with the lean group, and preincubation with ODQ further decreased the urethral relaxations (Fig. 2A; Table 2). No statistically significant differences for the pEC50 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 USM preparations produced a different pattern of response (Fig. 2B; Table 2). BAY 60-2770 promoted concentration-dependent urethral relaxations in the lean group that were rather potentiated by ODQ. In addition, the E_max and pEC50 values to BAY 41-2272 were 43% greater in the obese compared with lean mice (P < 0.05).

**Urethral Relaxations to Tadalafil and 8-Br-cGMP.** Cumulative addition of the phosphodiesterase-5 (PDE5) inhibitor tadalafil (0.0001–10 µM) or the permeable cGMP

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**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 or presence of 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 versus obese (ANOVA followed by Tukey test).
analog 8-Br-cGMP (0.003–100 μM) produced concentration-dependent urethral relaxations in the lean and obese groups. However, no statistically significant 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 minutes) 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 the obese group, however, GTN (10 μM, 10 minutes) 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 the obese group compared with the lean group ($P < 0.05$; Fig. 3). Incubation of urethral tissues with ODQ (10 μM) before the stimulation with BAY 60-2770 further elevated the cGMP levels in the lean but not in the obese group. The vehicle dimethylsulfoxide (0.22%) alone had no effect on the intracellular levels of cGMP in any condition.

**Long-Term Treatment with BAY 60-2770 on Urethral Relaxations, sGC Expression, and ROS Production.** Given that sGC activators reactivate the heme-oxidized enzyme in vascular diseases (Stasch et al., 2006) and ameliorate obesity-associated OAB (Leiria et al., 2013), we next examined
TABLE 2
Maximal responses ($E_{\text{max}}$) and potency (pEC50) values derived from concentration–response curves to BAY 41-2272 (0.0001–30 μM) and BAY 60-2770 (0.009–30 μM) in urethral smooth muscle from lean and obese mice in the presence and absence of ODQ (1 μM) and 8-Br-cGMP (1 μM), and in the absence of ODQ in the presence of 1,1,1-trifluoro-2-naphthylamine (TPA) (1 μM).

The present study shows an impairment of NO-cGMP-dependent USM relaxation in obese mice that is associated with enhanced ROS production and decreased protein levels of the 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 the urethra of obese mice.

Obesity is an important public health problem that greatly elevates the risk of urologic 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; Gisbarro et al., 2010; Lee et al., 2011; Leiria et al., 2012). However, no study has evaluated the potential implications of impairment of USM relaxations contributing to overall obesity-related micturition problems. Using a model of obesity-associated voiding dysfunction, we initially designed experiments to evaluate the in vitro USM reactivity to agents that interfere at different levels with the NO-sGC-cGMP-PDE5 signaling pathway. These agents included NO donors (acidified NaNO3, SNOG, and GTN), a sGC activator (BAY 41-2272), a PDE5 inhibitor (tadalafil), and a permeable cGMP analog (8-Br-cGMP).

The biologic effects of nitrates (GTN) and nitrosodones (SNOG) are attributable to NO formation via enzymatic or nonenzymatic bioactivation. Acidification of NaNO3 yields nitrous acid, which spontaneously decomposes to NO and other nitrogen oxides (Wang et al., 2002; Lundberg and Weitzberg, 2005). In the control group, NaNO3, SNOG, and GTN produced concentration-dependent urethral relaxations that were markedly reduced by oxidation of heme moiety (Fe3+) of sGC with ODQ, indicating a major role for cGMP in mediating these relaxing responses. Moreover, urethral relaxations to NaNO3, SNOG, and GTN were largely reduced in the obese mice in an ODQ-resistant manner, indicating that deficiency of cGMP production accounts for the impairment of USM relaxations in adiposity conditions. Accordingly, GTN markedly elevated the cGMP levels in urethral tissues of the lean but not the obese mice. It is unlikely that defects of urethral relaxation in obese mice rely on signal-transduction components downstream of cGMP generation, as the tadalafil and 8-Br-cGMP-induced responses remained unchanged between groups. We thus hypothesized that impairment of USM 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-subunits that convert guanosine triphosphate (GTP) to cGMP. Stimulators and activators of sGC have been developed over the past decade (Stasch et al., 2001, 2002). They comprise 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 the sGC enzyme. Similarly to endogenous ligand NO, sGC stimulators such as BAY 41-2272 increase sGC activity only when the heme iron is in its reduced state (Fe2+). On the other hand, sGC activators such as BAY 58-2667 and BAY 60-2770 preferentially induce sGC activation when the heme iron is in its oxidized state (Fe3+) 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 the obese mice.
group. Previous studies have reported the inhibitory actions of ODQ on aorta relaxations induced by BAY 41-2272 (Priviero et al., 2005; Teixeira et al., 2006). As opposed to BAY 41-2272, incubation with ODQ potentiated the urethral relaxations induced by the sGC activator BAY 60-2770 in lean mice. BAY 60-2770-induced relaxations were also greater in the obese compared with the lean mice. The enhanced functional responses by BAY 60-2770 are consistent with the higher cGMP levels in the 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 induce and accelerate heme loss from ferric sGC, or 2) they occupy the heme site in conditions where sGC is oxidized, avoiding degradation of the \( \alpha \)- and \( \beta \)-sGC subunits. BAY 58-2667-induced responses were greater in the aorta from spontaneously hypertensive rats and the mesocolon arteries from type 2 diabetic patients (Stasch et al., 2006). Additionally, the positive interaction of ODQ with the 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 the heme group of sGC is oxidized in the USM of obese mice.

A chronic state of oxidative stress is a hallmark of cardiovascular and endocrine metabolic diseases (Paravicini and Touyz, 2008). Oxidative stress appears to shift the balance to the NO-insensitive oxidized state, leading to downregulation of sGC, which may take place through \( \text{S} \)-nitrosylation (Sayed et al., 2007; Mayer et al., 2009). For instance, sGC protein expression is decreased in the 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;

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**Fig. 3.** Cyclic GMP content in urethral tissues from lean and obese mice. Tissues were stimulated with BAY 60-2770 (10 \( \mu \text{M} \)) alone or in the presence of ODQ (10 \( \mu \text{M} \)). Data are presented as mean \( \pm \) S.E.M. of three to four animals. \(*P < 0.05\) compared with respective nonstimulated lean group (basal). \( ^1 P < 0.05\) compared with lean in absence of ODQ.

**Fig. 4.** (A and B) Concentration–response curves to acidified nitrite solution (NaNO\(_2\)) 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 (\( E_{\text{max}} \)) values for all groups. Relaxations were calculated relative to the maximal changes from the contraction produced by phenylephrine (10 \( \mu \text{M} \)) in each urethral ring, which was taken as 100%. Data are presented as mean \( \pm \) S.E.M. (\( n = 6 \)). \(*P < 0.05\) lean versus obese (ANOVA followed by Tukey test).
Kagota et al., 2013). Reduction of protein levels of α1- and/or β1-subunits of sGC by oxidation of heme moiety (Fe3+) has also been reported in the 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 can 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 USM 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 the 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 likely accounts for the heme-oxidation of the β1-subunit of sGC in the urethra of obese mice.

The effect of BAY 60-2770 on sGC protein levels is hypothesized to be a result of the stabilization of the sGC subunits after BAY 60-2770 has bound 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. Additionally, the sGC activator BAY 58-2667 prevented the decrease in sGC protein levels induced by heme oxidants (Stasch et al., 2006; Hoffmann et al., 2009). However, the role of the redox state of sGC in regulating stability and protein levels remains unclear. It is interesting that a recent study showed that the 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 the bladder (Leiria et al., 2013) and in urethral tissues (present study). Two-week therapy with BAY 60-2770 did not significantly affect ROS levels in the bladder but rather normalized ROS levels in the urethra. The density of positive nerves for NO synthase is greater in the bladder neck and proximal urethra than in the bladder, and the NO-cGMP signaling pathway is more active in the urethra (Uckert and Kuczyk, 2011). Another previous study has shown that the urethra is more sensitive to ischemic injury than the bladder (Bratlavsky et al., 2001). Thus, it is possible that BAY 60-2770 causes a more efficient vasodilatation in the urethral vascular bed, ameliorating the blood perfusion and 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 urethral tissues and reduces ROS formation, resulting in amelioration of urethra dysfunction in high-fat fed obese mice. Our work highlights the possibility of targeting the USM to treat oxidative stress-related bladder dysfunction.

Fig. 5. (A) Representative images of Western blotting for β1-subunit of 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 in each group. *P < 0.05 in comparison with lean + vehicle.

Fig. 6. ROS levels through dye DHE-induced fluorescence in urethral tissues 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, 200×.
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References


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