British Journal of Pharmacology (2008) 153, 1659–1668
 0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Long-term nitric oxide deficiency causes muscarinic supersensitivity and reduces β_3-adrenoceptor-mediated relaxation, causing rat detrusor overactivity

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Background and purpose: Overactive bladder is a complex and widely prevalent condition, but little is known about its physiopathology. We have carried out morphological, biochemical and functional assays to investigate the effects of long-term nitric oxide (NO) deficiency on muscarinic receptor and β-adrenoceptor modulation leading to overactivity of rat detrusor muscle.
Experimental approach: Male Wistar rats received N^{ω}-nitro-L-arginine methyl ester (L-NAME) in drinking water for 7–30 days. Functional responses to muscarinic and β-adrenoceptor agonists were measured in detrusor smooth muscle (DSM) strips in Krebs–Henseleit solution. Measurements of [^{3}H]inositol phosphate, NO synthase (NOS) activity, [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding and bladder morphology were also performed.
Key results: Long-term L-NAME treatment significantly increased carbachol-induced DSM contractile responses after 15 and 30 days; relaxing responses to the β_3-adrenoceptor agonist BRL 37-344 were significantly reduced at 30 days. Constitutive NOS activity in bladder was reduced by 86% after 7 days and maintained up to 30 days of L-NAME treatment. Carbachol increased sixfold the [^{3}H]inositol phosphate in bladder tissue from rats treated with L-NAME. [^{3}H]QNB was bound with an apparent K_D twofold higher in bladder membranes after L-NAME treatment compared with that in control. No morphological alterations in DSM were found.
Conclusions and implications: Long-term NO deficiency increased rat DSM contractile responses to a muscarinic agonist, accompanied by significantly enhanced K_D values for muscarinic receptors and [^{3}H]inositol phosphate accumulation in bladder. This supersensitivity for muscarinic agonists along with reductions of β_3-adrenoceptor-mediated relaxations indicated that overactive DSM resulted from chronic NO deficiency.
British Journal of Pharmacology (2008) **153,** 1659–1668; doi:10.1038/bjp.2008.39; published online 25 February 2008

Keywords: detrusor smooth muscle; overactive bladder; inositol triphosphate; muscarinic receptors; trigone; β-adrenoceptors

Abbreviations: BRL 37-344, [±]-[*R*,*R*]-[4-(2-[(2-[3-chlorophenyl]-2-hydroxyethyl)amino]propyl)phenoxy]acetic acid; DSM, detrusor smooth muscle; [^{3}H]QNB, [^{3}H]quinuclidinyl benzilate; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO_x^-, nitrite/nitrate

Introduction

Urinary bladder function is regulated by a complex interaction of efferent and afferent fibres from the autonomic nervous system and somatic innervation. An imbalance between these two systems leads to disorders of urinary bladder reflexes, clinically expressed as symptoms of urgency, with or without urge incontinence (see Abrams *et al.*, 2006). This is usually accompanied by increases in frequency and nocturia, the components of the so-called overactive urinary bladder, as defined by the International Continence Society. Epidemiological studies have shown that overactive urinary bladder is widely prevalent, with an

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Received 5 September 2007; revised 5 December 2007; accepted 14 January 2008; published online 25 February 2008

incidence of 16.6% in a sample from Western Europe (Milsom *et al*., 2001), 17% of a sample from the USA (Stewart *et al*., 2001) and 18% in a South American sample population (Teloken *et al*., 2006).

The urinary bladder is divided into two components, namely bladder body that contains ureteral orifices and the detrusor smooth muscle (DSM), and the base consisting of the trigone, urethrovesical junction and the anterior bladder wall (Andersson and Arner, 2004). DSM in various animal species contains all muscarinic receptor subtypes, but M_2 and M_3 muscarinic receptors are predominant, with the ratio of 3:1 (Wang *et al*., 1995; Hedge and Eglen, 1999). Despite the predominance of M_2 receptors, it is the muscarinic M_3 receptors that are functionally responsible for urinary bladder contraction (Matsui *et al*., 2000; Igawa *et al*., 2004). In M_2, M_3 and M_2/M_3 double knockout mice, the M_2 receptor may have a role in indirectly mediating bladder contractions by enhancing the contractile response to M_3 receptor activation and minor M_2 receptor-mediated contractions may also occur (Ehlert *et al*., 2005). On the other hand, in another rodent study, stimulation of M_2 receptors may serve to inhibit β-adrenoceptor-mediated relaxation, which in turn leads to more efficient emptying of the bladder (Hegde *et al*., 1997). Relaxation of the urinary bladder is mediated by β-adrenoceptors that contributes to the collecting phase during bladder filling (Michel and Vrydag, 2006). The distribution of β-adrenoceptor subtypes in DSM varies significantly from species to species and, in rats, the relaxation responses are mediated by β_2- and β_3-adrenoceptor subtypes (Yamazaki *et al*., 1998). In patients with overactive urinary bladders, Harrison *et al*. (1987) reported DSM supersensitivity to muscarinic agonists and attenuation of nerve-mediated contractions, suggesting that the aetiology of detrusor hyper-reflexia reflects a state of postjunctional supersensitivity secondary to a partial parasympathetic denervation of the smooth muscle (German *et al*., 1995). On the other hand, little is known about the relaxing responses mediated by β-adrenoceptors in patients with overactive bladder (Nomiya and Yamaguchi, 2003).

Nitric oxide (NO) has been recognized as an important neurotransmitter in the lower urinary tract. Previous studies have shown the presence of NO synthases (NOSs) in urothelium and DSM in different animal species, including humans (see Andersson and Arner, 2004). Neuronal NOS expression was found in the nitrergic fibres of the submucosal surface and between muscle cells (Fathian-Sabet *et al*., 2001). A number of functional studies have also been carried out to explore the dependence on NO of relaxant responses in the DSM and NO has an inhibitory function in this tissue, although the sensitivity of DSM to NO depends markedly on the animal species (Morita *et al*., 1992; Persson *et al*., 1992; Masuda *et al*., 2002). More recently, the activation of the adenylate cyclase pathway by β-adrenoceptor agonists in bladder epithelial cells was shown to increase intracellular Ca^{2+} leading to NO release (Birder *et al*., 2002). Thus, studies of urinary bladder NOS isoform distribution together with the functional assays have provided evidence for the existence of the NO-cGMP signalling pathway in the DSM regulating basal tone, neurotransmission and blood flow.

Although the pathological alterations caused by long-term NO deficiency are well documented in cardiovascular diseases (Zatz and Baylis, 1998), there are no similar evaluations of the interactions of muscarinic receptors, adrenoceptors and NO in the lower urinary tract from rats, treated chronically with NOS inhibitors. Indeed, most of the functional assays have employed either acute administration of NOS inhibitors in the whole animal or *in vitro* addition to the isolated organ baths in electrically stimulated DSM. Therefore, in the present study, rats were treated chronically with the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) for up to 30 days, after which we examined the reactivity of DSM to both muscarinic and β-adrenoceptor agonists. Furthermore, we measured NOS activity, total [^{3}H]inositol phosphates and binding of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB), a muscarinic receptor antagonist, along with a morphometric analysis in bladder tissue, to explore the mechanisms underlying the effects of chronic NO deficiency in modulating reactivity of the detrusor muscle.

Methods

Animals and L-NAME treatment

All animal procedures and the experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation. Male Wistar rats (250–350 g) received L-NAME in the drinking water to give a daily intake of 20 mg per rat per day for 7, 15 and 30 days, as previously described (Ribeiro *et al*., 1992). Age-matched control rats received tap water alone. Systolic blood pressure was measured by using a modified tail-cuff method in conscious animals (Riado *et al*., 1999).

For the functional studies, the protocols resulted in six experimental groups of 5–10 rats each: (1) control, 7 days; (2) L-NAME, 7 days; (3) control, 15 days; (4) L-NAME, 15 days; (5) control, 30 days and (6) L-NAME, 30 days. For the protocols involving measurements of [^{3}H]inositol phosphate, NOS activity and [^{3}H]QNB binding, as well as bladder morphology, two experimental groups were employed, namely: (1) control, 30 days and (2) L-NAME, 30 days.

Functional studies

Rat isolated DSM. Rats were anaesthetized with halothane and exsanguinated. The urinary bladder was removed and sectioned horizontally at the level of the ureters. Isolated DSM strips were prepared and mounted in 10-ml organ baths containing Krebs–Henseleit solution with the following composition (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $NaHPO_4$, 25 $NaHCO_3$ and 11 glucose, pH 7.4, at 37 °C and bubbled with a gas mixture of 95% oxygen and 5% carbon dioxide. Changes in isometric force were recorded using a Power Lab v.5.0 system (Colorado Springs, CO, USA). The resting tension was adjusted to 2 g at the beginning of the experiments. The equilibration period was 60 min and the bathing medium was changed every 15 min.

Concentration–response curves. To verify the viability of the preparations, high extracellular K^+ solution (80 mM,

achieved by the replacement of NaCl in Krebs buffer with an equimolar concentration of KCl) was added to the organ baths at the end of the equilibration time. Cumulative concentration–response curves to the full muscarinic agonist, carbachol (10 nM to 10 μM), were constructed by using one-half log unit increments in the absence or presence of the Na^+ channel blocker tetrodotoxin (1 μM, 30 min) after 7, 15 and 30 days of L-NAME treatment. In another series of experiments carried out in naïve rats, L-NAME (100 μM, 30 min) was added *in vitro* prior to construction of concentration–response curves to carbachol. Concentration–response curves to isoprenaline (non-selective β-adrenoceptor agonist), metaproterenol (selective β_2-adrenoceptor agonist) and BRL 37-344 ([±]-[*R*,*R*]-[4-(2-[(2-[3-chlorophenyl]-2-hydroxyethyl)amino]propyl)phenoxy]acetic acid) selective β_3-adrenoceptor agonist) were also obtained in the presence of the α-adrenoceptor antagonist phentolamine (1 μM) and 17-β-estradiol (5 μM; extraneuronal uptake blocker). Relaxing responses were calculated as percentages of the maximal changes from the steady-state contraction produced by KCl (80 mM) in each tissue. The EC_{50} value for each agonist was determined as the molar concentration to produce 50% of the maximal relaxation elicited by the agonist in KCl-precontracted tissues.

Nonlinear regression analysis to determine the pEC_{50} was carried out using GraphPad Prism (GraphPad Software, San Diego, CA, USA) with the constraint that $\Phi = 0$. All concentration–response data were evaluated for a fit to a logistics function in the form

$$E = E_{max}/([1 + (10^c/10^x)^n] + \Phi$$

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 Φ is the response observed in the absence of added drug. The values of pEC_{50} data represent the mean ± s.e.mean. Maximal response (E_{max}) data were normalized to the wet weight of the respective urinary bladder strips, and the values of E_{max} were represented by milliNewton per milligram wet weight.

The series of experiments described below were carried out on tissues or plasma obtained from rats treated for 30 days with L-NAME in comparison with age-matched control rats.

Histological analysis. The rat isolated urinary bladder was removed and separated into trigone and detrusor, opened and fixed immediately in Bouin's fixative solution (37–40% formaldehyde, 750 ml picric acid and 50 ml glacial acetic acid) for 24 h. Tissues were then washed in water and embedded in 70% ethyl alcohol, according to conventional methods for light microscopy. Briefly, the pieces of the rat urinary bladder were embedded in paraffin and 5-μm sections were stained with haematoxylin–eosin. For quantitative analysis, a morphometric determination of the thickness of total and separate layers (muscular and submucosa) was performed in both trigone and detrusor in both control and L-NAME groups at 30 days using an ImageLab-2000 program (USP, São Paulo, Brazil).

Measurement of urinary bladder NOS activity. Animals were killed with halothane and urinary bladders were removed and cleaned in physiological saline. The tissue was homogenized in 50 mM Tris buffer 7.4 (100 ml) containing L-citrulline (1 mM), leupeptin (10 μg ml^{-1}), soyabean trypsin inhibitor (10 μg ml^{-1}), aprotinin (2 μg ml^{-1}) and phenylmethylsulphonyl fluoride (1 mM). Tissue homogenization was performed using Ultra-Turrax T25 (T-25; IKA Labortechnick, Staufen, Germany) in 5 ml buffer per g tissue (wet weight). Homogenates were centrifuged at 12 000 *g* for 10 min, and NOS activity was measured in the supernatant fractions (Hiki *et al*., 1992). Briefly, the supernatant fractions (50 μl) were incubated in a modified Tris buffer (50 mM, pH 7.4) containing $CaCl_2$ (91 mM), flavin adenine dinucleotide (10 μM), NADPH (1 mM), calmodulin (1 μg ml^{-1}) and tetrahydrobiopterin (100 μM), previously equilibrated at 37 °C for 5 min. Pharmacological controls of enzymatic activity were performed in parallel and consisted of either the omission of $CaCl_2$ and the addition of EGTA (1 mM) or the addition of L-NAME (1 mM) to the incubation medium. Aliquots (10 μl) of [^{3}H]L-arginine were used in each sample to give a final concentration of approximately 2×10^5 d.p.m. in a final volume of 100 μl. After 15 min the reaction was stopped by the addition of 1 ml ice-cold buffer (pH 5.4) containing HEPES (20 mM), EGTA (1 mM) and EDTA (1 mM) followed by vortex mixing. The samples were then applied to a 0.6 ml Dowex 50WX8-200 (ionic form: hydrogen, dry mesh 100–200; The Dow Chemical Company, St Louis, MO, USA) pre-equilibrated with the stopping buffer. [^{3}H]L-Citrulline was eluted and washed with 1 ml stopping buffer and radioactivity was determined by liquid scintillation counting. All measurements were made in duplicate. Protein concentration was determined using BSA as a standard. Activity was expressed as picomoles of L-citrulline per mg protein per minute. The values were corrected for by the amount of [^{3}H]L-citrulline found in the presence of L-NAME (1 mM) added exogenously.

Determination of plasma nitrite/nitrate levels. Arterial blood was centrifuged (8000 *g*) for 10 min, and the resulting plasma supernatant was stored at −80 °C. Plasma samples were ultrafiltered through microfilter cups (Microcon Centrifugal Filter Units, 10 kDa; Millipore, Bedford, MA, USA). The nitrite/nitrate (NO_x) concentration of the resulting filtrate solution was determined using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. This assay determines the total NO_x based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. After the conversion, the spectrophotometric measurement of nitrite is accomplished by using the Griess reaction. The resulting deep purple azo compound absorbs light at 540–550 nm.

Measurement of total [^{3}H]inositol phosphates. Bladders (70–90 mg) from both control and L-NAME-treated rats were allowed to equilibrate for 10 min in nutrient solution (pH 7.0–7.2) at 30 °C, under constant agitation. Thereafter, the tissues were incubated with 3 μCi of myo[^{3}H]inositol for 80 min, followed by addition of LiCl (10 mM). After 30 min, the tissues were incubated in the absence (basal level) and

the presence of carbachol (1 μM to 1 mM), and incubation was further continued for 40 min. When a muscarinic antagonist was used, it was added 10 min prior to incubation with carbachol (Abdalla *et al*., 2000). Tissues were washed three times with nutrient solution, transferred into 2 ml of methanol/chloroform (2:1 v/v) at 4 °C and homogenized (Ultra-Turrax T25 homogenizer, 9500 r.p.m.). Chloroform (0.62 ml) and H_2O (0.93 ml) were added to the homogenate, followed by centrifugation (2000 *g*, 10 min, 4 °C) to separate the aqueous and organic phases (Fox *et al*., 1985). Separation of total [^{3}H]inositol phosphates was performed as previously described (Ascoli *et al*., 1989) with slight modifications (Abdalla *et al*., 2000). Briefly, the aqueous layer was mixed with 1 ml anion-exchange resin (Dowex AG-X8, formate form, 200–400 mesh), allowed to equilibrate for 30 min at room temperature and centrifuged (1000 *g*, 5 min, 4 °C). The resin was then washed, sequentially, with 4 ml of myo-inositol and 2 ml of 5 mM sodium tetraborate plus 60 mM sodium formate. Next, 2 ml of 0.1 M formic acid/1 M ammonium formate was mixed with the resin and incubated for 30 min at room temperature. The total [^{3}H]inositol phosphates were eluted and placed in scintillation vials containing OptiPhase HiSafe 3 (Waltham, MA, USA). The amount of radioactivity was determined in a scintillation β-counter (LS 6500 IC; Beckman, Palo Alto, CA, USA). Total [^{3}H]inositol phosphates were expressed as disintegrations per minute per mg tissue.

Membrane preparation and binding assays. Isolated bladder tissues were minced and homogenized in 10 volumes of 25 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose, 0.5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, with an Ultra-Turrax homogenizer. The homogenate was centrifuged at 2000 *g* for 10 min. The supernatant was filtered through two layers of gauze and then centrifuged at 100 000 *g* for 1 h. The pellet was resuspended in 2 ml binding buffer (25 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride) using a Dounce homogenizer. All procedures were performed at 4 °C. The protein concentration of membrane preparations was determined according to the method of Bradford (1976) using BSA as a standard. Rat bladder membranes (200 μg protein ml^{-1}) were incubated with 0.05–8 nM [^{3}H]QNB in the absence (total binding) and in the presence (nonspecific binding) of atropine (1 mM) for 60 min at 30 °C in a final volume of 500 μl (Bricola *et al*., 2003). Specific binding was calculated as the difference between total and nonspecific binding. All experiments were performed in duplicate in membranes from control and L-NAME-treated rats. After incubation, the binding reaction was stopped by adding ice-cold phosphate-buffered saline and the mixture was filtered rapidly through a glass fibre filter (GF/B Whatman, Clifton, NJ, USA), under vacuum. The filters were washed three times with 1 ml ice-cold phosphate-buffered saline, partly dried under vacuum and placed in scintillation vials (OptiPhase, HiSafe 3). The amount of radioactivity was determined in a β-counter. Saturation binding data were analysed using the nonlinear least-square iterative curve-fitting program GraphPad Prism. A mathematical model for one or two sites was applied. The equilibrium K_D and the binding capacity (B_{max}) were determined from Scatchard analysis.

Statistical analysis. Data are expressed as mean ± s.e.mean of *n* experiments. The program Instat (GraphPad Software) and the SAS System for Windows (version 8.02) were used for statistical analysis. Two-way ANOVA followed by a Tukey test was performed. When appropriate, unpaired Student's *t*-test was used. $P<0.05$ was accepted as significant.

Drugs. Carbamylcholine chloride, isoprenaline, metaproterenol hemisulphate, L-NAME hydrochloride, BRL 37-344, phenylephrine, tetrodotoxin, L-citrulline, leupeptin, soyabean trypsin inhibitor, aprotinin, flavin adenine dinucleotide, NADPH, calmodulin, tetrahydrobiopterin, 17-β-estradiol, phenylmethylsulphonyl fluoride, EDTA, EGTA, lithium chloride and myo-inositol were obtained from Sigma Chemical Co. (St Louis, MO, USA). Halothane was provided by Cristalia (Itapira, São Paulo, Brazil). [^{3}H]myo-inositol (specific activity 47 Ci mmol^{-1}), [^{3}H]QNB (specific activity 37–44 Ci mmol^{-1}) and [^{3}H]L-arginine (specific activity 1.52 TBq mmol^{-1}) were supplied by Amersham Biosciences (Buckinghamshire, UK).

Results

Tail blood pressure and body weight in L-NAME-treated rats
Treatment with L-NAME produced a significant increase in tail blood pressure at 7, 15 and 30 days compared with their respective age-matched control group, without significantly changing the body weight (Table 1).

Determination of rat bladder NOS activity and plasma NO_x^- levels
Long-term L-NAME treatment reduced the calcium-dependent constitutive NOS activity in rat urinary bladder by 86% ($P<0.05$) at 7 days and this low level was maintained at 15 and 30 days of treatment (Figure 1). The plasma NO_x levels were significantly reduced in animals treated with L-NAME at 30 days (9.7 ± 1.9 μM; $n=5$) compared with the control group (21.8 ± 3.0 μM; $n=5$, $P<0.05$).

Table 1 Values of body weight and tail-cuff blood pressure (TCP) in control and rats treated chronically with L-NAME at 7, 15 and 30 days

	Body weight (g)		*TCP (mm Hg)*	
	Initial	*Final*	*Initial*	*Final*
7 days				
Control	219 ± 6	268 ± 7	117 ± 1	116 ± 2
L-NAME	225 ± 7	262 ± 2	114 ± 1	166 ± 3*
15 days				
Control	223 ± 3	282 ± 3	122 ± 2	118 ± 3
L-NAME	198 ± 2	268 ± 2	116 ± 3	170 ± 2*
30 days				
Control	228 ± 3	342 ± 5	120 ± 1	124 ± 2
L-NAME	217 ± 5	339 ± 5	114 ± 1	198 ± 1*

Data are the mean ± s.e.mean for $n=7$–10 animals.
*$P<0.0001$ compared with its respective age-matched control group.

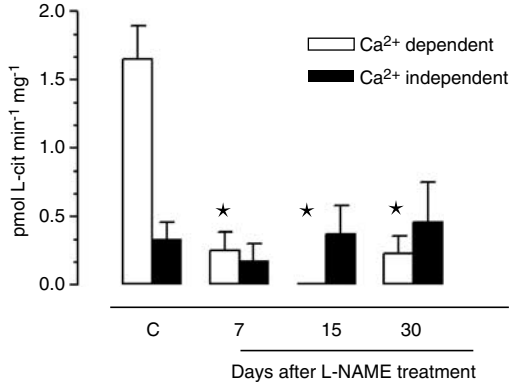

Figure 1 Nitric oxide synthase (NOS) activity in rat urinary bladder from control and N^{ω}-nitro-L-arginine methyl ester (L-NAME)-treated rats. Animals were treated with L-NAME (20 mg per rat per day) for 7, 15 and 30 days. Open columns show NOS activity under standard conditions for control and L-NAME groups, whereas closed columns show the NOS activity in the absence of Ca^{2+} and in the presence of EGTA. Activity is expressed as picomoles of citrulline per milligram protein per minute. Data are the mean ± s.e.mean of 5–6 animals. *$P<0.05$ compared with the control group (C).

Table 2 Values of potency (pEC_{50}) and maximal responses (E_{max}) obtained from concentration–response curves to carbachol in detrusor smooth muscle from control and L-NAME-treated rats after 7, 15 and 30 days

Treatment	*pEC_{50}*	E_{max} (mN per mg wet weight)
7 days		
Control	5.90 ± 0.03	2.54 ± 0.03
L-NAME	5.94 ± 0.01	2.59 ± 0.02
15 days		
Control	5.92 ± 0.03	2.92 ± 0.02
L-NAME	6.22 ± 0.04*	2.75 ± 0.12
30 days		
Control	6.09 ± 0.02	3.50 ± 0.10
L-NAME	6.82 ± 0.06*	3.40 ± 0.07

Data are the mean ± s.e.mean for $n=7$–10 animals.
*$P<0.0001$ compared with the respective age-matched control group.

Concentration–response curves to carbachol and KCl

Carbachol (10 nM to 10 μM) produced concentration-dependent contraction responses in the DSM. The *in vitro* addition of L-NAME to the organ bath (100 μM, 30 min) modified neither the potency (pEC_{50}: 5.96 ± 0.03 vs 5.81 ± 0.05) nor the maximal responses (E_{max}: 2.67 ± 0.30 vs 2.78 ± 0.22 mN per mg wet weight) for control and L-NAME treated rats, respectively ($n=5$).

Long-term L-NAME treatment for 7 days caused no alterations in the pEC_{50} and E_{max} values for carbachol, whereas a significant leftward shift at the pEC_{50} level was found after 15 and 30 days of L-NAME treatment (2.0- and 5.5-fold, respectively; $P<0.001$) in comparison with their respective age-matched control groups. No changes of maximal response values were observed in any of the groups. Data are summarized in Table 2 and Figure 2. Preincubation of rat DSM with tetrodotoxin (1 μM, 30 min) modified neither the potency nor maximal responses for carbachol in all groups ($n=7$–10; data not shown).

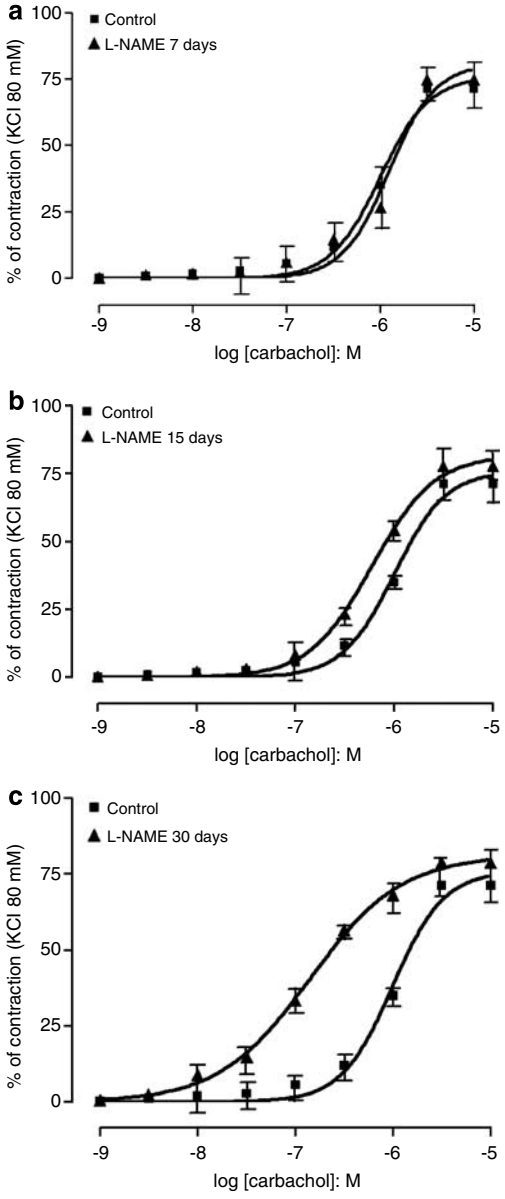

Figure 2 Concentration–response curves to carbachol in the rat isolated detrusor smooth muscle from control and L-NAME (20 mg per rat per day)-treated rats at 7 (**a**), 15 (**b**) and 30 days (**c**). Data are the mean ± s.e.mean for 7–10 animals.

Non-cumulative addition of the depolarizing agent KCl (20–200 mM) induced DSM contractions in both control and L-NAME-treated rats at 30 days, but no significant differences in the contractile responses between these groups were found ($n=6$; data not shown).

Concentration–response curve to β-adrenoceptor agonists

The non-selective β-adrenoceptor agonist isoprenaline (0.1 nM to 10 μM; $n=6$), the β_2-adrenoceptor agonist metaproterenol (0.01–100 μM; $n=5$) and the β_3-adrenoceptor agonist BRL 37-344 (0.1 nM to 100 μM; $n=6$) produced concentration-dependent DSM relaxations in KCl-precontracted strips with a rank order for their potency of BRL 37-344 > isoprenaline > metaproterenol (Table 3). Treatment

Table 3 Potency (pEC_{50}) and maximal response (E_{max}) values for isoprenaline (non-selective β-adrenoceptor agonist), metaproterenol (selective β_2-adrenoceptor agonist) and BRL 37-344 (selective β_3-adrenoceptor agonist) in rat isolated detrusor smooth muscle precontracted with KCl (80 mM)

	pEC50		E_{max} *(mN per mg wet weight)*	
	Control	*L-NAME*	*Control*	*L-NAME*
Isoprenaline	6.38 ± 0.19	6.48 ± 0.06	105.72 ± 4.86	103.03 ± 4.98
Metaproterenol	4.70 ± 0.24	4.89 ± 0.08	63.18 ± 6.24	64.74 ± 2.32
BRL 37-344	7.22 ± 0.16	6.68 ± 0.16*	78.78 ± 5.18	72.81 ± 4.15

Data represent mean ± s.e.mean for 5–6 animals.
*$P<0.05$ compared with its age-matched control group.

with L-NAME for 30 days caused a significant reduction ($P<0.05$) of the pEC_{50} for the β_3-adrenoceptor agonist BRL 37-344, about 3.5-fold. The pEC_{50} values for isoprenaline and metaproterenol were not affected by 30 days of L-NAME treatment (Figure 3 and Table 3). The E_{max} values for all these agonists were not significantly modified by the chronic L-NAME treatment in comparison with age-matched control rats (Table 3). Acute treatment of rats with L-NAME (100 mg kg^{-1}, i.v., 30 min) had no effect on the pEC_{50} and E_{max} values for BRL 37-344 (7.27 ± 0.12 and 80.5 ± 7.4%, respectively; $n=6$) in comparison with animals injected with saline (7.02 ± 0.21 and 74.5 ± 13.9%, respectively; $n=6$).

Measurement of total [^{3}H]inositol phosphate

Treatment with L-NAME for 30 days did not significantly modify the basal levels of total [^{3}H]inositol phosphates in the rat urinary bladder (78.1 ± 15.6 d.p.m. per mg tissue; $n=25$) compared with control group (69.3 ± 5.3 d.p.m. per mg tissue; $n=20$). Incubation of urinary bladders with carbachol caused a concentration-dependent increase in the total [^{3}H]inositol phosphates in these tissues from L-NAME-treated rats that was markedly higher than that in control rats (Figure 4a). The maximum response was reached with 100 μM carbachol. The magnitude of this maximum response was sixfold higher ($P<0.05$) in urinary bladder from L-NAME-treated rats than in control animals (Figure 4a). Addition of the selective M_3/M_1 receptor antagonist *p*-fluoro-hexahydro-sila-diphenidol (100 nM, 5 min) to the incubation bath had no effect on the basal levels of total [^{3}H]inositol phosphates in both groups (not shown), but it significantly reduced the [^{3}H]inositol phosphate levels induced by carbachol in both groups (Figure 4b).

Binding of [^{3}H]QNB in rat urinary bladder membranes

The binding of [^{3}H]QNB to rat bladder membranes was specific and saturable in both groups (Figure 5). The apparent K_D for [^{3}H]QNB in the group treated with L-NAME was twofold higher ($P<0.05$) than in the control group (2.16 ± 0.34 and 0.99 ± 0.14 nM, respectively), whereas no significant differences between L-NAME and control groups were found for the binding capacity (B_{max}) (221.3 ± 46.7 and 234.9 ± 47.5 fmol per mg protein, respectively).

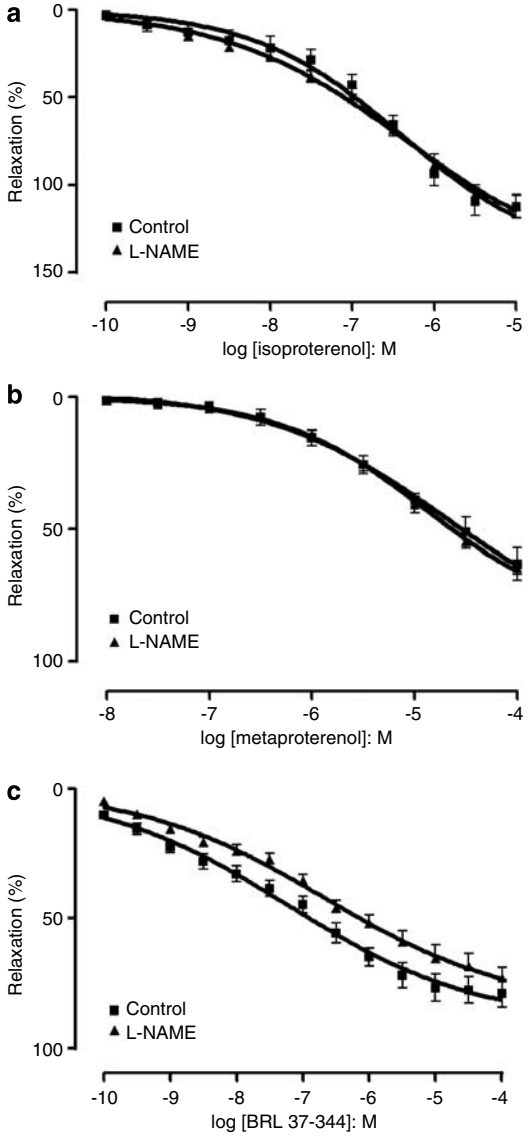

Figure 3 Concentration–response curves to isoprenaline (**a**), metaproterenol (**b**) and BRL 37-344 (**c**) in the rat isolated detrusor smooth muscle from control and L-NAME (20 mg per rat per day)-treated rats for 30 days. Data are the mean ± s.e.mean for $n=5$–6 animals.

Histological analysis

The measurement of the thickness of rat urinary bladder revealed that L-NAME treatment at 30 days caused no alterations in the thickness of DSM submucosa and muscular layers compared with control animals (Table 4). In the trigone smooth muscle, however, L-NAME treatment significantly increased the thickness of the muscular layer without changing the thickness of the submucosa layer (Table 4).

Discussion and conclusions

The present study is the first to show that long-term NO deficiency increases the contractile responses mediated by muscarinic agonists in rat DSM and is accompanied by

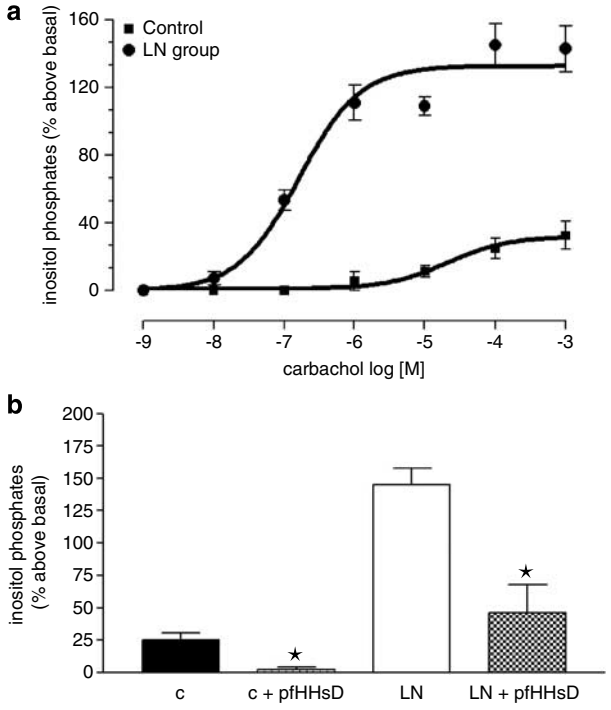

Figure 4 (**a**) Dose-dependent increase of total [^{3}H]inositol phosphate levels in response to carbachol (10^{-9}–10^{-3} M) in urinary bladder from control and L-NAME (20 mg per rat per day; 30 days)-treated rats. (**b**) Carbachol (100 μM)-induced increase of total [^{3}H]inositol phosphate levels in control (C) and L-NAME-treated rats (LN) in the absence and the presence of the muscarinic receptor antagonist *p*-fluoro-hexahydro-sila-diphenidol (pfHHSiD; 100 μM). Each point and vertical line represents the mean ± s.e.mean for $n = 20$ each group. *$P < 0.05$ compared with its respective C and LN groups.

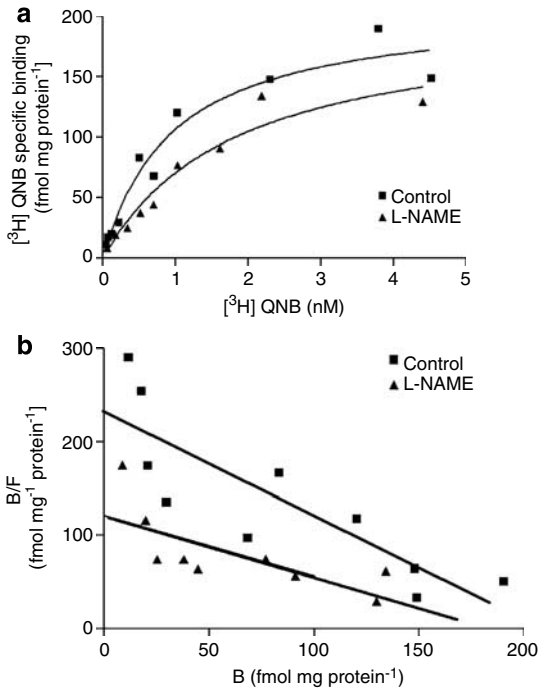

Figure 5 (**a**) Saturation curves and (**b**) Scatchard plot analysis of specific [^{3}H]quinuclidinyl benzilate (QNB) binding to muscarinic receptor in rat bladder membranes from control and L-NAME (20 mg per rat per day)-treated rats for 30 days. Results are representative for $n = 3$–4, performed in duplicate.

Table 4 Morphometric analysis of rat isolated detrusor and trigone smooth muscle in both control and L-NAME-treated groups at 30 days, shown as thickness of muscular and serosal layers

Layers	*Detrusor (μm)*		*Trigone (μm)*	
	Control	*L-NAME*	*Control*	*L-NAME*
Total	1194 ± 73.09	1098 ± 67.7	706 ± 19.6	861 ± 21.8*
Muscular	612 ± 37.2	659 ± 32.2	527 ± 24.3	678 ± 30.5*
Serosal	547 ± 66.4	495 ± 56.1	177 ± 12.5	183 ± 17.1

Data are the mean ± s.e.mean for $n = 10$ animals.
*$P < 0.05$ compared with its age-matched control group.

significant enhancement of the apparent K_D of muscarinic receptors and [^{3}H]inositol phosphate levels in the urinary bladder. Additionally, L-NAME treatment reduced the DSM relaxations mediated by β_3-adrenoceptors. Thus, the supersensitivity for muscarinic agonists along with the reduction of β_3-adrenoceptor-mediated relaxations strongly suggests that prolonged NO deficiency leads to an overactive DSM.

Clinically, an overactive bladder is manifested by symptoms of urgency, usually accompanied by increases in frequency and nocturia. Evidence indicates that overactive urinary bladder is related to increased sensitivity of DSM to muscarinic receptor agonists (Chapple *et al*., 2002; Stevens *et al*., 2007). Although it has been accepted that the nitrergic fibres supplying the urinary bladder and hence the local NO release provide relaxation of the urethral outlet during micturition (Mamas *et al*., 2003), the interactions between supersensitivity for muscarinic agonists and NO deficiency in eliciting overactive bladder have been poorly explored. Thus, we initially carried out functional assays to explore the influence of long-term NO inhibition on the contractile responses of DSM mediated by muscarinic receptors. Our findings showed that long-term L-NAME treatment (7–30 days) caused time-dependent increases in the potency (pEC_{50}) for the full muscarinic agonist carbachol that achieved maximum leftward shift (fivefold increase) at 30 days of treatment. The failure of the voltage-gated sodium channel blocker tetrodotoxin to modify the DSM supersensitivity to carbachol in L-NAME-treated rats confirms that this phenomenon is taking place at a postjunctional level.

The maximum inhibition of bladder NOS activity (approximately 86%) was observed after only 7 days of treatment with L-NAME, a time at which no functional alterations to muscarinic agonists were detected, indicating that supersensitivity to muscarinic agonists takes place only after a prolonged NO deficiency. Accordingly, *in vitro* addition of L-NAME to the organ bath (100 μM) had no effect on the concentration–response curves to carbachol. In our study, the removal of Ca^{2+} ions (and addition of EGTA) reduced the urinary bladder NOS activity by 80%, in both control and L-NAME groups, confirming that conversion of [^{3}H]arginine to [^{3}H]citrulline was mainly due to Ca^{2+}-dependent constitutive NOS, with no involvement of iNOS. In fact, the iNOS isoform has been detected only in urinary bladder undergoing inflammatory conditions (Poljakovic *et al*., 2001; Felsen *et al*., 2003).

It is well established that ACh stimulates muscarinic M_3 receptors to cause direct DSM contraction (Abrams *et al*.,

2006) via interaction with G_q to elicit phosphoinositide hydrolysis and hence generation of the second messenger inositol triphosphate that activates the inositol triphosphate receptor to release Ca^{2+} from internal stores and subsequently facilitating the entry of Ca^{2+} via plasma membrane cation channels (Bolton, 2006). In our study, stimulation *in vitro* of urinary bladders with carbachol increased the levels of [^{3}H]inositol phosphate in the control group, an effect clearly potentiated by 30 days of treatment with L-NAME, further supporting our data of DSM supersensitivity to carbachol in the functional assays. High levels of extracellular K^+ depolarize the cell membrane and activate L-type Ca^{2+} channels, leading to elevation of the intracellular Ca^{2+} concentration, which in turn activates contractile proteins (Andersson and Arner, 2004). In our study, the contractile responses to exogenous KCl in L-NAME-treated rats were not modified in comparison with untreated rats, indicating that increased inward movement of Ca^{2+} does not contribute to the bladder supersensitivity during chronic NO deficiency.

The functional supersensitivity in the urinary bladder of L-NAME group to carbachol could reflect changes at the receptor level (density and/or affinity) or beyond the receptor related to the transducer proteins (Kenakin, 1993). Our data clearly showed that long-term L-NAME treatment induced increases in [^{3}H]inositol phosphate in rat DSM.

Our data with [^{3}H]QNB-binding assays in rat urinary membranes revealed that K_D value increased twofold in the L-NAME group than the control group, whereas no differences between both groups for B_{max} were found. This indicates that chronic L-NAME treatment increases the receptor affinity without affecting the muscarinic receptor density. Using [^{3}H]*N*-methylscopolamine-binding assays in rat urinary bladder, recent studies showed that treatment of the animals with the M_1/M_3 muscarinic receptor antagonist oxybutynin causes an increase in the K_D values without changing the B_{max} (Oki *et al*., 2004, 2006). In our study, the possibility that chronic L-NAME treatment could act by antagonizing muscarinic receptors at the level of urinary bladder is excluded, as supersensitivity to carbachol in L-NAME-treated rats was observed.

The relaxation of detrusor muscle via β-adrenoceptors is thought to contribute to urine storage during bladder filling (Andersson and Arner 2004). In our study, isoprenaline, metaproterenol and BRL 37-344 (non-selective-, β_2- and β_3-selective adrenoceptor agonists, respectively) produced concentration-dependent DSM relaxations with a rank order of potency for BRL 37-344 > isoprenaline > metaproterenol. Thus, our data indicate a functional importance for the β_3-adrenoceptor in mediating the relaxant response in rat urinary bladder. In previous studies, a great variability of potency for non-selective (isoprenaline), β_2-(terbutaline, procaterol) and β_3-selective (BRL 37-344, SR58611A, CGP 12177, CL316243) adrenoceptor agonists in rat DSM was found (Yamazaki *et al*., 1998; Longhrust and Levendusky, 1999; Moore *et al*., 2002; Frazier *et al*., 2006). These discrepancies at pEC_{50} level may reflect the different methodological conditions employed, such as measurement of relaxations under resting tension or under precontraction, agent used to precontract tissues (KCl, carbachol or both together), rat strain (Sprague–Dawley and Wistar) and presence or absence of extraneuronal uptake blockers. Nevertheless, in our study, 30 days of treatment with L-NAME caused a 3.5-fold reduction in the potency of BRL 37-344 (without affecting those for isoprenaline and metaproterenol), thus suggesting that β_3-adrenoceptor-mediated DSM relaxations involve NO-dependent mechanisms. Again, such reductions of BRL 37-344-induced DSM relaxations by chronic L-NAME take place after prolonged NO deprivation as acute L-NAME treatment failed to affect the relaxing responses. No previous study has investigated the coupling of β_3-adrenoceptors and NO in DSM. However, in other tissues such as endothelium of rat aorta and human coronary arteries, as well as human myocardium, the activation of β_3-adrenoceptors by BRL 37-344 or CL316243 induces cellular responses by mechanisms involving NOS activation (Gauthier *et al*., 1998; Trochu *et al*., 1999; Dessy *et al*., 2004) via phosphorylation or eNOS translocation (Brixius *et al*., 2004).

Nitric oxide has many physiological functions, including the control of proliferation and differentiation of smooth muscle cells, where NO acts as an inhibitor of cell proliferation (Jeremy *et al*., 1999). Gene deletion for neuronal NOS causes bladder hypertrophy, dysfunctional urinary outlet and increased urinary frequency (Burnett *et al*., 1997). In rats with partial urethral obstruction, NO has been shown to inhibit the growth of bladder smooth muscle cells (Johansson *et al*., 2002). In our present study, we have carried out morphometric studies of the urinary bladder in control and L-NAME-treated groups in an attempt to explain our functional assays. We found that long-term L-NAME treatment did not affect the thickness of DSM layer, but rather increased the thickness of the trigone smooth muscle layer. As the density of NOS immunoreactive nerves is more prominent in the trigone (and urethra) than in the DSM (Persson *et al*., 1995), it is plausible to suggest that chronic NO deficiency at the level of trigone (and/or urethra) triggers a persistent contraction leading in turn to an overactivity in the DSM. Accordingly, clinical studies have reported that urethra obstruction secondary to a benign prostatic hyperplasia can potentially lead to overactive bladder (Novara *et al*., 2006).

In conclusion, our data obtained with long-term NO deficiency show the development of DSM supersensitivity to muscarinic agonists via increases in the receptor affinity (K_D values) and levels of [^{3}H]inositol phosphate, which is accompanied by reductions of β_3-adrenoceptor-mediated DSM relaxations. Therefore, chronic L-NAME administration to rats constitutes a new paradigm to study the mechanisms of the physiopathology of the overactive bladder and to test novel pharmacological agents for management of this disorder.

Acknowledgements

Fabiola ZT Monica and E Antunes thank Fundação de Amparo à Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Científico e Tecnológico. We thank Dr Maria AB Teixeira, Ronaldo Victorino de Barros and Dr Mirian Trevisan (Departments of Pathologic Anatomy,

and Histology & Pathology, Faculty of Medicine, PUC-Campinas, São Paulo; and MULTIPAT, Brazil) for their great efforts facilitating the histology analysis presented in this study.

Conflict of interest

The authors state no conflict of interest.

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