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Increased Rho-kinase-mediated prostate contractions associated with impairment of  $\beta$ -adrenergic-cAMP-signaling pathway by chronic nitric oxide deficiency

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## ABSTRACT

Impairment of nitric oxide (NO) – cyclic GMP signaling pathway is likely to contribute to human benign prostate hyperplasia (BPH). In the present study we have used a model of chronic NO synthesis inhibition to evaluate the functional alterations of prostate smooth muscle (PSM) machinery, and involvement of Rho-kinase pathway. Wistar rats were treated with the NO inhibitor N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg/day; 4 weeks), after which contractile responses to phenylephrine ( $\alpha_1$ -adrenoceptor agonist; 1 nM to 100  $\mu$ M), carbachol (muscarinic agonist; 1 nM to 1 mM) and  $\alpha,\beta$ -methylene ATP (P2X receptor agonist; 1–10  $\mu$ M), as well as to electrical-field stimulation (EFS; 1–32 Hz) were evaluated. PSM relaxations to isoproterenol (non-selective  $\beta$ -adrenoceptor agonist, 0.1 nM to 10  $\mu$ M) and sodium nitroprusside (NO donor, 1 nM to 10 mM) were also evaluated. The ratio prostate weight/body weight was 22% greater ( $P < 0.05$ ) in L-NAME compared with control group. The PSM contractions to phenylephrine, carbachol and  $\alpha,\beta$ -methylene ATP were higher in L-NAME ( $E_{\max}$ :  $3.85 \pm 0.25$ ,  $3.52 \pm 0.35$  and  $2.03 \pm 0.2$  mN, respectively) compared with control group ( $E_{\max}$ :  $3.08 \pm 0.17$ ,  $2.37 \pm 0.18$  and  $1.57 \pm 0.18$  mN, respectively). The PSM contractions induced by EFS were also significantly greater in L-NAME group. Prior incubation with the Rho-kinase inhibitor Y27632 (1  $\mu$ M) fully reversed the enhanced contractions to phenylephrine and carbachol. Isoproterenol-induced PSM relaxations were 34% lower in L-NAME group, which was associated with reduced levels of cAMP in prostate tissue. The relaxations to sodium nitroprusside remained unaltered in L-NAME group. In summary, chronic NO deficiency leads to increased Rho-kinase-mediated PSM contractile responses accompanied by impairment of  $\beta$ -adrenergic-cAMP-signaling pathway.

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## 1. Introduction

Prostate smooth muscle (PSM) is densely innervated by excitatory sympathetic and parasympathetic autonomic fibers, the activation of which results in the release of the neurotransmitters noradrenaline and acetylcholine, respectively (McVary et al., 1998; Witte et al., 2008). Noradrenaline acting in post-junctional  $\alpha_1$ -adrenoceptors ( $\alpha_1$ -AD) together with acetylcholine acting in muscarinic M<sub>3</sub> receptors (mAChR) produce PSM contractions mainly via Ca<sup>2+</sup>-IP<sub>3</sub> signaling-dependent mechanisms (Michel and Vrydag, 2006; Ventura et al., 2002). Purinergic excitatory neurotransmission via functional ligand-gated purinergic

receptors (P2 × 1) also produces PSM contractions (Ventura et al., 2003). Studies have also identified nitric oxide (NO) produced by nitrergic nerves as an important inhibitory neurotransmitter involved in PSM relaxations in animal and human prostate (Di Iulio et al., 1997; Hedlund, 2005; Kedia et al., 2008). Prostate-generated NO is mainly produced via neuronal NO synthase (nNOS) present in nerves and ganglia in the transition zone, and in prostate epithelium (Bloch et al., 1997). Endothelial NO synthase (eNOS) has also been found in endothelium and epithelial cells (Gradini et al., 1999). In benign prostatic hyperplasia (BPH) and aging, the reductions of nitrergic innervation and NO-mediated relaxations, together with increased PSM tone have been reported to contribute to PSM dysfunction (Aikawa et al., 2001; Bloch et al., 1997; Dey et al., 2012).

Changes in the RhoA/Rho-kinase signaling pathway account for the increased peripheral vascular resistance that leads to hypertension (Rao et al., 2013). In the lower urinary tract, including prostate

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(Christ and Andersson, 2007), both  $\alpha_1$ -adrenoceptor and muscarinic receptor-mediated PSM contractile responses are regulated by RhoA/Rho-kinase  $\text{Ca}^{2+}$ -sensitization pathway, as demonstrated in animal (Lam et al., 2013; Saito et al., 2011; White et al., 2011, 2013) and human tissues (Rees et al., 2003; Strittmatter et al., 2011; Takahashi et al., 2007). Elevated RhoA/Rho-kinase signaling has also been shown to play a role in BPH (Adam, 2003; Gur et al., 2011). Evidence shows the existence of a functional antagonism between the Rho-kinase pathway and the NO/cGMP pathway in vasculature, which has been clearly demonstrated in the rat model of chronic NO inhibition (Ikegaki et al., 2001; Ito et al., 2004; Kataoka et al., 2002; Seko et al., 2003). No previous study, however, has evaluated the effects of chronic NO inhibition in PSM and its modulation by Rho-kinase. We hypothesized that Rho-kinase activation under chronic NO deficiency contributes to alterations of PSM contractile machinery. Thus, the aim of this study was to investigate the rat PSM reactivity to  $\alpha_1$ -AD, muscarinic and P2X receptor activation in rats undergoing chronic NO blockade, and its modulation by Rho-kinase. Since activation of  $\beta$ -AD leads to PSM relaxations due to activation of adenylate cyclase–cAMP signaling pathway (Carmena et al., 1997; Kalodimos and Ventura, 2001), and that activation of  $\alpha_1$ -AD causes phosphorylation of  $\beta_2$ -AD in the human prostate (Hennenberg et al., 2011), this study also aimed to evaluate the  $\beta$ -AD-mediated PSM relaxations and cAMP levels in chronic NO-deficient rats.

## 2. Material and methods

### 2.1. Animals

All experimental procedures were conducted in accordance with institutional guidelines, and they were approved by the Ethical Principles in Animal Research by the Brazilian College for Animal Experimentation (COBEA). Male wistar rats (initially weighing 250–300 g) were provided by Central Animal House Services (CEMIB) of State University of Campinas (UNICAMP, São Paulo). Animals were housed 5 per cage on 12 h light–dark cycle, and fed a standard chow diet with water ad libitum.

### 2.2. L-NAME treatment

Rats that received L-NAME at the dose of 20 mg/rat/day for four weeks, given in the drinking water, according to previous studies (Medeiros et al., 1995). Briefly, L-NAME was dissolved in the drinking water at a concentration of 400 mg/L to give a daily intake of 20 mg/rat/day. The volume of water drunk by each rat was approximately 50 ml/rat/day. Control animals received tap water alone. Systolic blood pressure was recorded weekly by tail-cuff system using a PowerLab 400™ data acquisition system (Software Chart, version 4.2, AD Instruments, MA, USA).

### 2.3. Prostate preparation and concentration–response curves

Animals were anesthetized with halothane and exsanguinated. Prostate was removed and immediately placed in chilled Krebs solution of the following composition (mM): NaCl, 118;  $\text{NaHCO}_3$ , 25; glucose, 5.6; KCl, 4.7;  $\text{KH}_2\text{PO}_4$ , 1.2;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.17 and  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2.5. Prostate was dissected and each strip was mounted under resting tension of 5 mN in 4-ml organ chambers containing Krebs solution at 37 °C (pH 7.4) and continuously bubbled with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Isometric force was recorded using a PowerLab 400™ data acquisition system (Software Chart, version 6.0, AD Instrument, MA, USA). The tissues were allowed to equilibrate for 1 h before starting the experiments. Cumulative concentration–response curves to phenylephrine (PE;  $\alpha_1$ -adrenoceptor agonist; 1 nM to 100  $\mu\text{M}$ ), carbachol

(CCh; muscarinic receptor agonist; 1 nM to 1 mM) and non-cumulative curve to  $\alpha, \beta$ -methylene ATP (purinergic P2X agonist; 1, 3 and 10  $\mu\text{M}$ ) were constructed by using one-half log unit increments. Each prostate strip was used to construct only one concentration–response curve. The maximal response ( $E_{\text{max}}$ ) and potency ( $\text{pEC}_{50}$ ) were determined for all tested agonists.  $E_{\text{max}}$  was represented by mN (contraction protocols). Relaxing responses to isoproterenol (nonselective  $\beta$ -adrenoceptor agonist; 1 nM to 10  $\mu\text{M}$ ) and sodium nitroprusside (NO donor; 1 nM to 10 mM) were also performed in PSM preparations. Relaxing responses were calculated as percentages of the maximal changes from the steady-state contraction produced by CCh (10  $\mu\text{M}$  to isoproterenol relaxation) and PE (10  $\mu\text{M}$  to sodium nitroprusside) in each tissue. The  $E_{\text{max}}$  and  $\text{pEC}_{50}$  were also determined.

### 2.4. Electrical-field stimulation (EFS)-induced prostate contractions

Electrical-field stimulation (EFS) was applied in prostate strips placed between two platinum electrodes (3 mm diameter) connected to a Grass S88 stimulator (Astro-Med Industrial Park). Frequency–response curves (1–32 Hz) were elicited by stimulating the tissues for 10 s with pulses of 1 ms width at 50 V, with 2 min interval between stimulations.

### 2.5. Measurements of cAMP and cGMP levels

Prostate strips were equilibrated for 30 min in warmed and oxygenated Krebs solution. To quantify cAMP levels, tissues were stimulated for 4 min with isoproterenol (1  $\mu\text{M}$ ) in the absence and in the presence of the adenylate cyclase inhibitor SQ 22,536 (100  $\mu\text{M}$ , 30 min). Frozen tissues were separately pulverized, homogenized in trichloroacetic acid (TCA, 5% wt/vol), and centrifuged for 10 min at 4 °C at 1500g. The supernatant was collected, and the pellet was dried and weighted. TCA was extracted from the supernatant with three washes of water saturated ether. Preparation of tracer, samples, standards and incubation with antibody were performed as described in the Kit instructions (Cayman Chemical Cyclic AMP or Cyclic GMP EIA Kit, Ann Arbor, MI, USA). The assays were performed in triplicate, and the pellet weight was used to normalize the data, which were expressed as pmol/mg tissue.

### 2.6. Statistical analysis

All data are expressed as means  $\pm$  S.E.M. ( $n$ ). The program InStat (GraphPad software) was used for statistical analysis. One-way ANOVA and unpaired Student's  $t$ -test was used to evaluate the results.  $P < 0.05$  was accepted as significant.

## 3. Results

### 3.1. Tail-cuff pressure, body weight, prostate weight and prostate/body weight ratio

Four-week treatment with L-NAME (20 mg/rat/day) produced a significant increase ( $P < 0.001$ ) in tail-cuff pressure compared with control group (Table 1). A small (but significant) reduction ( $P < 0.05$ ) in body weight after L-NAME treatment was observed. The prostate weight did not change significantly between both groups. The prostate/body weight ratio was 22% greater ( $P < 0.01$ ) in L-NAME compared with control group (Table 1).

### 3.2. Concentration–response curves to PE, CCh, $\alpha,\beta$ -methylene ATP and EFS

Addition of PE (1 nM to 100  $\mu$ M) produced concentration-dependent prostate contractions in both control and chronic L-NAME groups (Fig. 1A). However, the  $E_{max}$  was significantly greater in L-NAME compared with control group, whereas the  $pEC_{50}$  to this  $\alpha_1$ -adrenoceptor agonist did not change between groups (Table 2). The muscarinic agonist CCh (1 nM to 1 mM) also produced concentration-dependent contractile responses in control and L-NAME groups (Fig. 1B). The  $E_{max}$  and  $pEC_{50}$  values for CCh were greater in L-NAME compared with control group (Fig. 1B, Table 2).

In separate prostate preparations, in vitro addition of L-NAME (100  $\mu$ M, 30 min) to the organ baths did not significantly affect PSM contractions induced by PE ( $pEC_{50}$ :  $6.42 \pm 0.06$  and  $6.35 \pm 0.07$ ;  $E_{max}$ :  $3.22 \pm 0.25$  and  $3.26 \pm 0.22$  mN, for control and L-NAME, respectively;  $n=5$ ) or CCh ( $pEC_{50}$ :  $5.70 \pm 0.15$  and  $5.72 \pm 0.10$ ;  $E_{max}$ :  $2.16 \pm 0.29$  and  $2.08 \pm 0.21$  mN, for control and L-NAME, respectively;  $n=8$ ).

**Table 1**

Tail-cuff pressure (TCP), body weight (BW), prostate weight (PW) and BW/PW ratio after 8 weeks of treatment with  $N^o$ -nitro-L-arginine methyl ester (L-NAME, 20 mg/rat/day, 4 weeks) in rats.

	Control	L-NAME
TCP (mmHg)	118 $\pm$ 2	198 $\pm$ 6.1 <sup>a</sup>
BW (g)	462 $\pm$ 6	431 $\pm$ 5 <sup>b</sup>
PW (mg)	381 $\pm$ 18	434 $\pm$ 27
PW/BW ratio (mg/g)	82.5 $\pm$ 3.8	100.6 $\pm$ 5.7 <sup>b</sup>

Data are the mean  $\pm$  S.E.M. ( $n=10$ –11 rats).

<sup>a</sup>  $P < 0.001$  compared with respective control group.

<sup>b</sup>  $P < 0.01$ .

Non-cumulative addition of the P2X receptor agonist  $\alpha,\beta$ -methylene ATP (1, 3 and 10  $\mu$ M) caused concentration-dependent PSM contractions that were greater in preparations from chronic L-NAME compared with those from control rats (Fig. 1C,  $n=5$ –6).

In the control group, EFS (1 to 32 Hz) produced frequency-dependent PSM contractions, which were significantly reduced ( $P < 0.05$ ) by pre-incubation (30 min) with the non-selective  $\alpha$ -adrenoceptor antagonist phentolamine (10  $\mu$ M; 43% reduction at 8 Hz stimulation), the muscarinic receptor antagonist atropine (10  $\mu$ M; 50% reduction at 8 Hz stimulation) and the purinergic P2X receptor antagonist PPADS (30  $\mu$ M; 37% reduction at 8 Hz stimulation). A cocktail of phentolamine, atropine and PPADS reduced by  $> 70\%$  the EFS-induced contractions ( $n=4$ ). In rats treated with L-NAME, EFS-induced PSM contractions were significantly greater at

**Table 2**

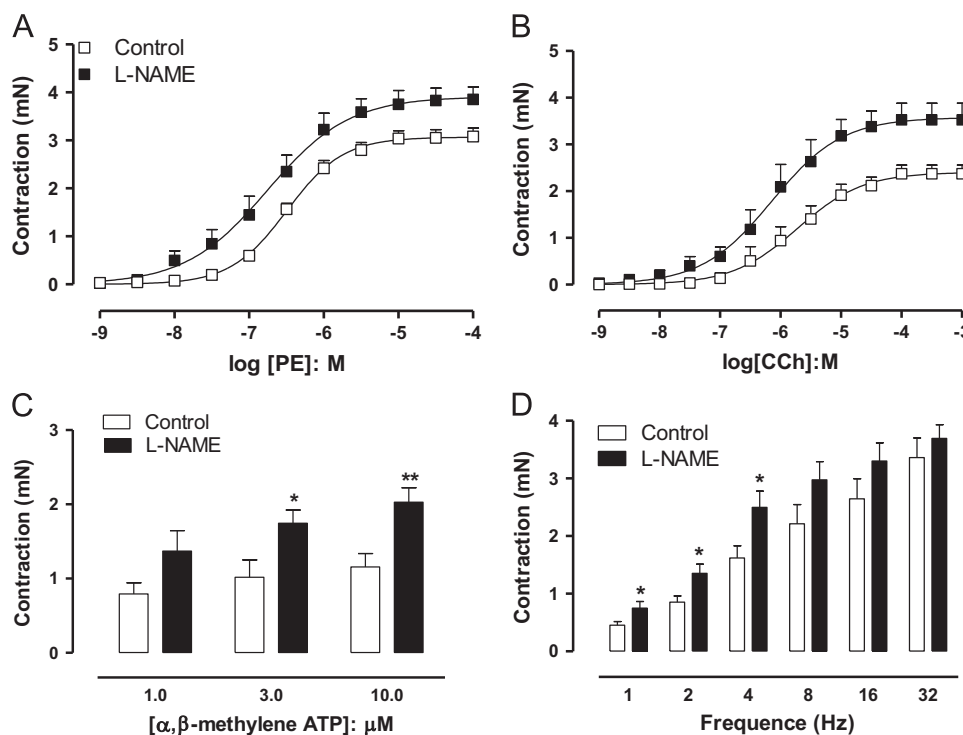
Potency ( $pEC_{50}$ ) and maximal responses ( $E_{max}$ ) of cumulative concentration–response curves to phenylephrine (PE;  $\alpha_1$ -adrenoceptor agonist), carbachol (CCh; non-selective muscarinic receptor agonist), isoproterenol (ISO; non-selective  $\beta$ -adrenoceptor agonist) and sodium nitroprusside (SNP, NO donor compound) in prostate smooth muscle from control and L-NAME-treated rats. Prostate relaxations induced by ISO and SNP were expressed relative to the maximal changes from the contraction produced by carbachol, which was taken as 100%.

Agents	Parameters	Control	L-NAME
PE ( $n=9$ –11)	$pEC_{50}$	6.49 $\pm$ 0.04	6.75 $\pm$ 0.11
	$E_{max}$	3.08 $\pm$ 0.17	3.85 $\pm$ 0.25 <sup>a</sup>
CCh ( $n=5$ )	$pEC_{50}$	5.72 $\pm$ 0.12	6.14 $\pm$ 0.11 <sup>a</sup>
	$E_{max}$	2.37 $\pm$ 0.18	3.52 $\pm$ 0.35 <sup>b</sup>
ISO ( $n=5$ )	$pEC_{50}$	7.62 $\pm$ 0.06	7.62 $\pm$ 0.08
	$E_{max}$	67.3 $\pm$ 5.56	44.6 $\pm$ 3.75 <sup>a</sup>
SNP ( $n=5$ )	$pEC_{50}$	5.29 $\pm$ 0.10	5.14 $\pm$ 0.15
	$E_{max}$	72.6 $\pm$ 4	75.1 $\pm$ 2.6

Data are the mean  $\pm$  S.E.M. ( $n$ =number of rats in each group).  $N^o$ -nitro-L-arginine methyl ester (L-NAME; 20 mg/rat/day, 4 weeks).

<sup>a</sup>  $P < 0.05$ .

<sup>b</sup>  $P < 0.001$  compared with respective control group.



**Fig. 1.** Concentration–response curves to phenylephrine (PE, 1 nM to 100  $\mu$ M; A), carbachol (CCh, 1 nM to 1 mM; B),  $\alpha,\beta$ -methylene ATP (1, 3 and 10  $\mu$ M; C) and electrical-field stimulation (EFS, 1–32 Hz; D) in rat prostate smooth muscle preparations obtained from control and L-NAME ( $N^o$ -nitro-L-arginine methyl ester)-treated rats (20 mg/rat/day, 4 weeks). Data represent the mean  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.001$  compared with control group.

the frequencies of 1, 2 and 4 Hz compared with control group ( $P < 0.05$ ; Fig. 1D).

### 3.3. Relaxant responses to isoproterenol and cAMP levels

The non-selective  $\beta$ -adrenoceptor agonist isoproterenol (1 nM to 10  $\mu$ M) produced concentration-dependent PSM relaxations in both control and L-NAME groups (Fig. 2A).  $E_{max}$  to isoproterenol was significantly lower ( $P < 0.05$ ) in L-NAME compared with control group, whereas no differences between groups were found at the level of  $pEC_{50}$  (Table 2).

In control group, incubation of prostate tissues with isoproterenol (1  $\mu$ M) elevated by 13-fold the cAMP levels above baseline ( $P < 0.001$ ;  $n=3$ ). A significant reduction ( $P < 0.05$ ) in the cAMP levels was found in prostate tissues obtained from L-NAME-treated rats (Fig. 2B). Prior incubation of prostate tissues with the adenylate cyclase inhibitor SQ22535 (100  $\mu$ M) markedly reduced the isoproterenol-induced cAMP elevations in both control and L-NAME groups.

### 3.4. Effect of the Rho-kinase Y27632 in the PSM hypercontractility of L-NAME-treated rats

PSM preparations from both control and L-NAME treated rats were pre-treated with Y27632 (1  $\mu$ M, 30 min) before performing concentration–response curves to PE or CCh.

In concentration–response curves to PE in control group, pretreatment with Y27632 significantly reduced the  $pEC_{50}$  (shift of 4.3) with any effects in the  $E_{max}$  value (Fig. 3A and B; Table 3). In L-NAME group, Y27632 produced a markedly higher right displacement in PE-induced contractions (shift of 7.8) with a concomitant normalization of the  $E_{max}$  value (Fig. 3A and B; Table 3).

In concentration–response curves to CCh in control group, Y27632 did not significantly affect  $pEC_{50}$  (shift of 1.4;  $P=0.5$ ) and  $E_{max}$  values ( $P=0.078$ ) (Fig. 3C and D; Table 3). However, in L-NAME

group, Y27632 produced a marked right displacement (shift of 3.5) with a significant reduction in  $E_{max}$  (Fig. 3C and D; Table 3).

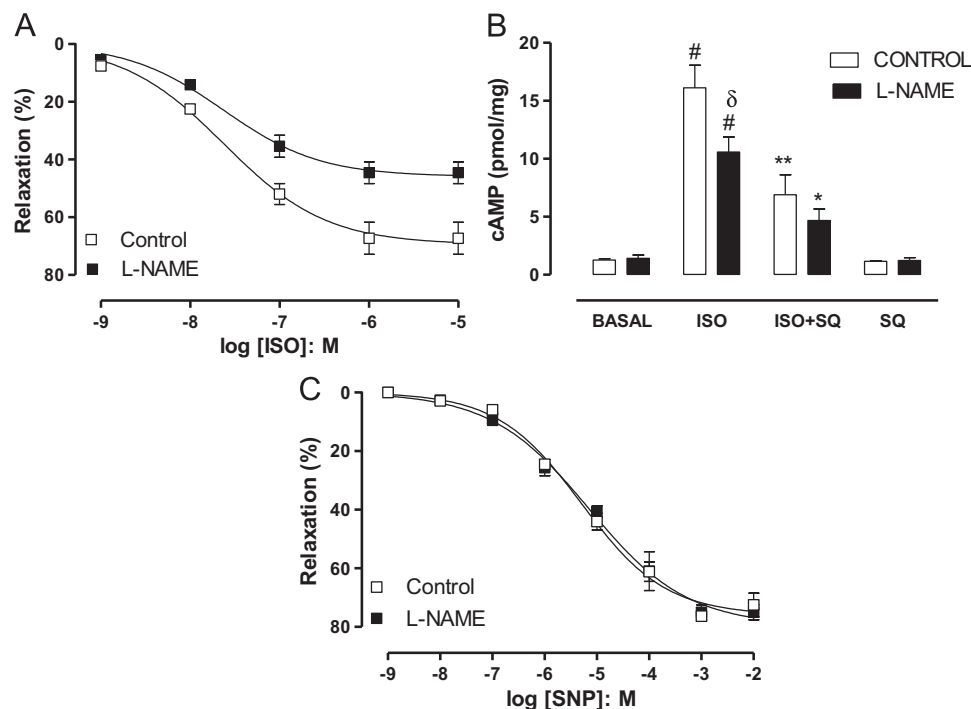
### 3.5. PSM relaxant responses to sodium nitroprusside

The NO donor sodium nitroprusside (1 nM to 10 mM) produced concentration-dependent PSM relaxations in both control and L-NAME groups. However, no significant differences between groups were found for this compound at the levels of  $pEC_{50}$  or  $E_{max}$  (Fig. 2C, Table 2). Basal cGMP levels in prostate tissues of L-NAME group were 43% lower ( $P < 0.05$ ) than the control group ( $4 \pm 0.4$  and  $7 \pm 1$  fmol/mg, respectively), confirming the efficacy of chronic L-NAME treatment.

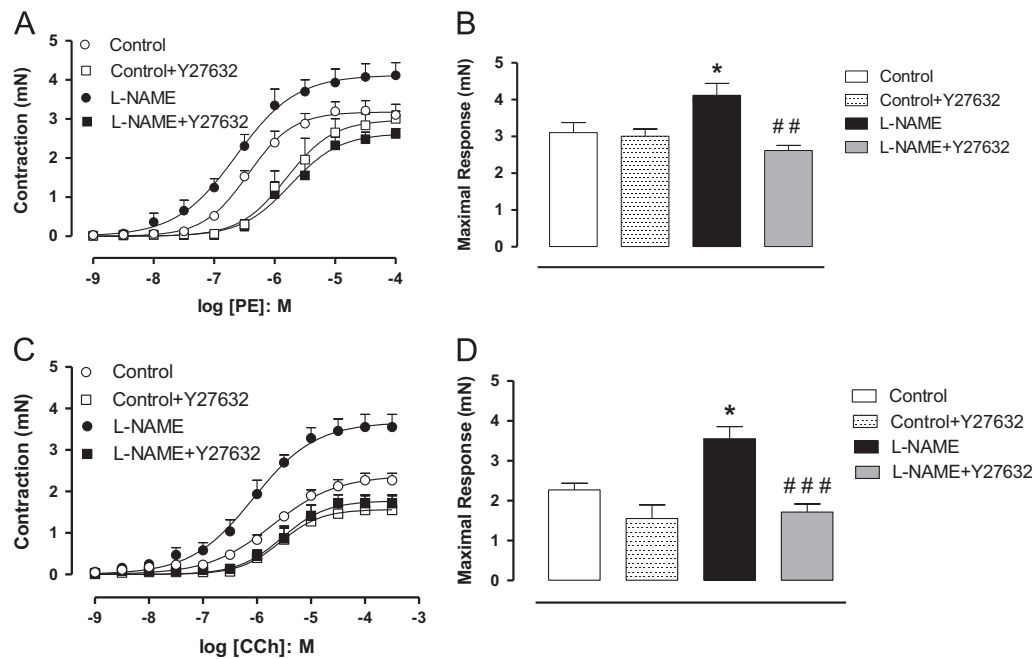
## 4. Discussion

This study shows that chronic NO deficiency causes greater PSM contractions to  $\alpha_1$ -AD and muscarinic activation, which are fully prevented by the Rho-kinase inhibitor Y27632, indicating an important role for the Rho-kinase signaling pathway in mediating the prostate hypercontractility. Significant reductions of  $\beta$ -AD-induced PSM relaxations and cAMP levels were also observed in chronic NO-deficient rats, which may contribute to the state of prostate hypercontractility.

The cardiovascular alterations caused by long-term NO deficiency are well documented. Chronic NO deficiency also promotes bladder overactivity as result of increased contractile responses to muscarinic receptor activation and reduced  $\beta_3$ -AD-mediated relaxations, which is restored by stimulation of soluble guanylate cyclase (Mónica et al., 2008, 2011). Most of functional studies addressing to examine the role of NO on PSM reactivity have employed in vitro addition of NO inhibitors to isolated organ baths in electrically-stimulated prostate preparations (Najbar-Kaszkiel et al., 1997; Takeda et al., 1995). However, no similar studies attempting to



**Fig. 2.** Concentration–response relaxing curves to the non-selective  $\beta$ -adrenoceptor agonist isoproterenol (ISO; A) and cAMP contents (B) in prostate smooth muscle obtained from control and L-NAME ( $N^G$ -nitro-L-arginine methyl ester)-treated rats (20 mg/rat/day, 4 weeks). Panel C shows the concentration–response curves to the nitric oxide donor sodium nitroprusside (SNP). For measurement of cAMP contents, prostate tissues were stimulated with ISO (1  $\mu$ M) in absence and presence of the adenylate cyclase inhibitor SQ22,536 (100  $\mu$ M, 30 min). Data represent the mean  $\pm$  S.E.M.  $^{\#}P < 0.001$  compared with respective basal levels;  $^{\delta}P < 0.05$  compared with ISO-stimulated prostate tissues in control group;  $^*P < 0.05$  and  $^{**}P < 0.001$  compared with respective ISO-stimulated tissues in absence of SQ22,536.



**Fig. 3.** Prostate smooth muscle contractions in response to the  $\alpha_1$ -adrenoceptor agonist phenylephrine (PE; A and B) and muscarinic receptor agonist carbachol (CCh; B and D) in the absence and in the presence of the Rho-kinase inhibitor Y27632 (1  $\mu$ M) in control and L-NAME ( $N^G$ -nitro-L-arginine methyl ester)-treated rats (20 mg/rat/day, 4 weeks). Data represent the means  $\pm$  S.E.M. \* $P$  < 0.05 compared with control group, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 compared with L-NAME group without Y27632.

**Table 3**  
Potency (pEC<sub>50</sub>) and maximal responses (E<sub>max</sub>) of cumulative concentration–response curves to phenylephrine (PE) and carbachol (CCh) in prostate smooth muscle preparations in the absence and in the presence of the Rho kinase inhibitor Y27632 (1  $\mu$ M) in control and L-NAME-treated rats.

Groups	PE			CCh		
	pEC <sub>50</sub>	Shift	E <sub>max</sub>	pEC <sub>50</sub>	Shift	E <sub>max</sub>
Control	6.43 ± 0.07		3.10 ± 0.27	5.71 ± 0.09		2.27 ± 0.17
Control+Y27632	5.80 ± 0.12 <sup>a</sup>	4.3	3.00 ± 0.19	5.56 ± 0.18	1.4	1.55 ± 0.34
L-NAME	6.63 ± 0.10		4.12 ± 0.32 <sup>b</sup>	6.07 ± 0.11		3.55 ± 0.31 <sup>a</sup>
L-NAME+Y27632	5.74 ± 0.10 <sup>c</sup>	7.8	2.61 ± 0.13 <sup>d</sup>	5.53 ± 0.11 <sup>e</sup>	3.5	1.71 ± 0.20 <sup>c</sup>

Data are the mean  $\pm$  S.E.M. ( $n=5-6$  rats).

Shift, arithmetic multiple for change in potency

$N^G$ -nitro-L-arginine methyl ester (L-NAME; 20 mg/rat/day, 4 weeks).

<sup>a</sup>  $P$  < 0.001 compared with respective control group.

<sup>b</sup>  $P$  < 0.05.

<sup>c</sup>  $P$  < 0.001 compared with L-NAME group.

<sup>d</sup>  $P$  < 0.01.

<sup>e</sup>  $P$  < 0.05.

evaluate the PSM contractile responses to  $\alpha_1$ -AD, muscarinic and  $P2 \times 1$  activation from rats treated chronically with NO inhibitors have been carried out. Studies addressing to evaluate the relaxant machinery secondary to activation of cAMP and cGMP in prostate in conditions of chronic NO deprivation have also been neglected. In the present study, we show that PSM contractions induced by PE, CCh and  $\alpha, \beta$ -methylene ATP are significantly greater in chronic L-NAME compared with control group, as evaluated at both potency (pEC<sub>50</sub>) and/or efficacy (E<sub>max</sub>). Electrically-stimulated contractile PSM responses, which results mainly from the release of noradrenaline, acetylcholine and ATP (Najbar-Kaszkiel et al., 1997), were also greater in L-NAME group, reinforcing our data with direct receptor activation. Interestingly, however, addition of L-NAME to the organ bath (100  $\mu$ M) failed to affect CCh- and PE-induced contractions, indicating that long-term rather than acute NO inhibition accounts for the functional PSM alterations.

Activation of  $\alpha_1$ -AD and muscarinic elicits phosphoinositide (PI) hydrolysis and hence generation of the second messenger IP<sub>3</sub> that activates the IP<sub>3</sub> receptor to release Ca<sup>2+</sup> from internal stores

(Somlyo and Somlyo, 2003). The state of Ca<sup>2+</sup>-dependent phosphorylation of myosin light chain kinase (MLCK) is further regulated by myosin phosphatase, an enzyme tightly regulated by RhoA and its downstream target Rho-kinase, as evidenced in different smooth muscle types, including prostate (Christ and Andersson 2007; Saito et al., 2011; Takahashi et al., 2007). A physiological antagonism between Rho-kinase pathway and NO has been clearly described in vascular smooth muscle (Kolluru et al., 2014), but no such antagonism has been explored in PSM. In our study, a Rho-kinase inhibitor Y27632 fully prevented the enhanced PE and CCh-induced PSM contractions in L-NAME group, restoring the E<sub>max</sub> to control levels. This finding indicates that RhoA/Rho-kinase pathway plays a key role in the enhanced agonist-induced PSM contractions in conditions of prolonged NO inhibition. Of note, the increased  $\alpha, \beta$ -methylene ATP-induced contractions in rat detrusor, trigonal and urethral smooth muscles have also been associated with Rho-kinase pathway (Teixeira et al., 2007).

Prostate relaxations mediated by activation of  $\beta$ -AD-cAMP play a key role to the maintenance of the physiological tonus of PSM



(Michel, 2011). Recently, the selective  $\beta_3$ -AD agonist mirabegron was shown to counteract the human and rabbit PSM contractions induced by EFS and  $\alpha_1$ -AR activation, highlighting a potential use of this compound in BPH treatment (Calmasini et al., 2014). In our study, the PSM relaxant response to the non-selective  $\beta$ -AD receptor agonist isoproterenol was significantly lower in L-NAME-treated rats, which was accompanied by lower stimulated cAMP production in the prostate tissues. A previous study in human prostate reported that activation of  $\alpha_1$ -adrenoceptor leads to  $\beta_2$ -adrenoceptor phosphorylation and consequent desensitization (Hennenberg et al., 2011). It is likely therefore that the increased contractile responses to EFS in L-NAME group reflect an augment in noradrenaline release, leading to  $\beta$ -AD phosphorylation with consequent impairment in cAMP production. Further investigations will be needed to confirm this hypothesis. Nitric oxide has been shown to downregulate the noradrenaline release from sympathetic neurons (Schwarz et al., 1995), which may also explain our data showing augmented EFS-induced contractions in L-NAME group.

The NO-cGMP-PDE5 pathway also plays an important role in producing PSM relaxations, the impairment of which results in BPH (Hedlund, 2005). Accordingly, PDE5 inhibitors have been successfully used to treat this condition (Oelke et al., 2012). In our study, the PSM relaxations to the NO donor sodium nitroprusside were not significantly changed by the chronic L-NAME treatment, indicating that the intracellular relaxant machinery downstream NO remains unaffected by chronic L-NAME treatment.

Benign prostate enlargement and the resulting bladder outflow obstruction in humans have been linked with lower urinary tract symptoms, including urgency with or without urge-incontinence, and increases in frequency and nocturia. Rats under prolonged L-NAME administration develop a marked arterial hypertension, but to our knowledge the reactivity of PSM in animal models of hypertension other than chronic L-NAME has not been explored in literature. In addition, a small (but significant) increase in prostate weight/body weight (PW/BW) ratio was detected in L-NAME group. However, this increased PW/BW ratio may not be considered a relevant prostate enlargement when compared with the classical model of testosterone in rats (Oudot et al., 2012). Thus, it is unlikely that increased PW/BW ratio in our study contributes to the PSM dysfunction in chronic L-NAME-treated rats.

In summary, rats treated chronically with the NO synthesis inhibitor L-NAME present PSM dysfunction as evidenced by the greater Rho kinase-dependent contractions to  $\alpha_1$ -AD and muscarinic receptor activation along with lower relaxations secondary to  $\beta$ -AD-cAMP pathway activation.

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