# Hydrochlorothiazide Potentiates Contractile Activity of Mouse Cavernosal Smooth Muscle

Thiago Gagliano-Jucá, MD, PhD,[1] Mauro Napolitano, PhD,[2] Fernanda Del Grossi Ferraz Carvalho, PhD,[3] Rafael Campos, MPhil,[3] Fabíola Zakia Mónica, PhD,[3] Mário Angelo Claudino, PhD,[3] Edson Antunes, PhD,[3] Anibal Gil Lopes, MD, PhD,[1] and Gilberto De Nucci, MD, PhD[2,3]

## ABSTRACT

**Introduction:** Hydrochlorothiazide has a negative influence on penile erection but little is known about the mechanism(s) involved.

**Aims:** To characterize the effects of this diuretic on mouse corpus cavernosum (CC) smooth muscle in vitro and ex vivo.

**Methods:** CC strips of C57BL/6 mice (12−16 weeks old) were mounted in organ baths containing Krebs-Henseleit solution and tissue reactivity was evaluated. Expression of genes encoding diuretic targets and enzymes involved in penile erection were evaluated by polymerase chain reaction.

**Main Outcome Measures:** Stimulation-response curves to phenylephrine (10 nmol/L−100 μmol/L) or to electrical field stimulation (1−32 Hz) were constructed, with or without hydrochlorothiazide. Strips of CC from mice after long-term hydrochlorothiazide treatment (6 mg/kg/day for 4 weeks) with or without amiloride (0.6 mg/kg/day for 4 weeks) in vivo also were studied. Nitric oxide and Rho-kinase pathways were evaluated.

**Results:** Hydrochlorothiazide (100 μmol/L) increased the maximum response to phenylephrine by 64% in vitro. This effect was unaffected by the addition of indomethacin (5 μmol/L) but was abolished by N(ω)-nitro-L-arginine methyl ester (100 μmol/L). Hydrochlorothiazide (100 μmol/L) potentiated electrical field stimulation-induced contraction in vitro, but not ex vivo. Long-term treatment with hydrochlorothiazide increased the maximum response to phenylephrine by 60% and resulted in a plasma concentration of 500 ± 180 nmol/L. Amiloride (100μmol/L) caused rightward shifts in concentration-response curves to phenylephrine in vitro. Long-term treatment with hydrochlorothiazide plus amiloride did not significantly increase the maximum response to phenylephrine (+13%). Reverse transcriptase polymerase chain reaction did not detect the NaCl cotransporter in mouse CC. Hydrochlorothiazide did not change Rho-kinase activity, whereas amiloride decreased it in vitro and ex vivo (approximately 18% and 24% respectively). A 40% decrease in Rock1 expression also was observed after long-term treatment with hydrochlorothiazide plus amiloride.

**Conclusion:** Hydrochlorothiazide potentiates contraction of smooth muscle from mouse CC. These findings could explain why diuretics such as hydrochlorothiazide are associated with erectile dysfunction.



**Key Words:** Corpus Cavernosum; Erectile Dysfunction; Adrenergic Mechanism; Diuretics

Received November 24, 2015. Accepted February 21, 2016.

[1]Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil;

[2]Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo (USP), Sao Paulo, SP, Brazil;

[3]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil



http://dx.doi.org/10.1016/j.esxm.2016.02.003

## INTRODUCTION

Hydrochlorothiazide (HTZ) and other thiazide diuretics are commonly used as monotherapy or in combination with other drugs in the treatment of arterial hypertension. Although their primary site of action is in the kidney, the major hypotensive effect of thiazides during long-term administration is due to vasodilation rather than saluresis or loss of free water.[1−3] Thiazides cause vasorelaxation in vitro and in vivo, and the mechanisms proposed for this action are not related to their natriuretic effects (inhibition of the NaCl cotransporter).[4−8] For instance,

vasodilation of the human brachial artery in vivo induced by HTZ also occurs in patients with Gitelman syndrome, which is characterized by the presence of a non-functional thiazide-sensitive NaCl cotransporter.[9] Thiazides cause alterations in ion channel homeostasis and HTZ-induced relaxations appear to be mediated by $Ca^{2+}$-activated $K^+$ channels.[7,10,11] For example, the relaxant effect of HTZ in guinea pig isolated mesenteric small arteries was associated with a decrease in intracellular calcium concentration ($[Ca^{2+}]_i$).[7,12]

Thiazide and thiazide-like diuretics can cause sexual dysfunction in humans, especially with high doses of diuretics and hypertension.[13–19] Nitric oxide (NO) released from non-adrenergic, non-cholinergic nitrergic nerve endings and from the endothelium is the most important mediator of corpus cavernosum (CC) smooth muscle relaxation.[20] In contrast, noradrenaline, released from adrenergic nerves, is the major mediator of penile CC contraction.[21] Thus, an imbalance between the contractile and relaxant mechanisms of non-vascular smooth muscle could contribute significantly to the observed erectile dysfunction (ED) after thiazide therapy.[22]

## AIMS

Using CC strips from mice, the effect of HTZ in vitro on the contraction of CC smooth muscle induced by phenylephrine or electrical field stimulation (EFS) was investigated. Because amiloride is often coadministered with HTZ to prevent the occurrence of hypokalemia,[23,24] its possible interaction on HTZ effects in vitro also was evaluated. The effects of long-term treatment in vivo with HTZ, with or without amiloride, on the contractility of CC smooth muscle ex vivo were investigated. The involvement of the NO and the Rho-kinase (ROCK) pathways in HTZ-induced altered CC contractility was studied. Expression of genes encoding the main target transporter for HTZ (NaCl cotransporter) and amiloride (epithelial sodium channel [ENaC]) also was assessed in the CC of mice.

## METHODS

### Animals

Male adult (3–4 months old) C57BL/6 mice (25–30 g) were obtained from the animal care facility of the State University of Campinas (Campinas, SP, Brazil) and used in this study. Animals were housed in temperature-controlled facilities on a 12-hour light-and-dark cycle with food and water ad libitum. All experimental procedures were conducted in accordance with institutional guidelines and were approved by the ethical principles in animal research adopted by the Brazilian College for Animal Experimentation.

### Mouse CC Preparation

The animals were rendered unconscious by inhalation of $CO_2$ and then decapitated. The penises were surgically removed and immediately placed in chilled Krebs solution (NaCl 118 mmol/L, $NaHCO_3$ 25 mmol/L, glucose 5.6 mmol/L, KCl 4.7 mmol/L, $KH_2PO_4$ 1.2 mmol/L, $MgSO_4$ 1.17 mmol/L, and $CaCl_2$ 2.5 mmol/L). Cavernosal strips were obtained by dissection of the tunica albuginea and surrounding connective tissues. Each penis resulted in two strips, one for each CC, that were vertically suspended between two metal hooks in 10-mL organ baths containing Krebs solution at 37°C continuously bubbled with a mixture of 95% $O_2$ and 5% $CO_2$ (pH = 7.4). One hook was connected to a force transducer (ADInstruments, Colorado Springs, CO, USA) and the other acted as a fixed attachment point. The resting tension was adjusted to 4 mN and CC strips were allowed to equilibrate for 60 minutes.[25–27] Changes in isometric force were recorded using a PowerLab 4/30 data acquisition system (Software Chart, version 7.0; ADInstruments).

### Concentration-Response Curves

Cumulative concentration-response curves for phenylephrine (10 nmol/L–100 μmol/L) were constructed for the CC strips in the absence (control) or presence of HTZ (10, 30 and 100 μmol/L) and amiloride (100 μmol/L) added 30 minutes before phenylephrine. The NO synthase inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L) or the cyclooxygenases inhibitor indomethacin (5 μmol/L) was used to evaluate any influence of NO or prostaglandins on HTZ effects in cavernosal contractility.[28–30] Each strip was used for only one concentration-response curve. The possible pharmacologic interaction between HTZ and amiloride in the contractile response to phenylephrine also was evaluated by incubating CC strips with HTZ (100 μmol/L) in the presence or absence of amiloride (100 μmol/L). To evaluate cholinergic-mediated relaxation, cumulative concentration-response curves were obtained by adding acetylcholine (ACh; 0.001–10 μmol/L) to CC strip pre-contracted with U46619 (9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin F2α; 100 nmol/L)[31] in the absence (control) or presence of HTZ (100 μmol/L). All concentration-response data were evaluated for fit to a logistics function and non-linear regression analysis was used to determine the parameters maximal response and log half-maximal effective concentration ($EC_{50}$) using GraphPad Prism (GraphPad Software, San Francisco, CA, USA).

### Electrical Field Stimulation

For EFS experiments, CC strips were placed between two platinum ring electrodes. EFS was carried out at 50 V for 10 seconds at a range of frequencies from 1 to 32 Hz in square-wave pulses (1-ms pulse width) using a Grass S88 stimulator (Astro-Med, West Warwick, RI, USA). Frequency-response curves to EFS were constructed to evaluate changes in basal tone of strips to EFS in the absence and presence of L-NAME (100 μmol/L) and/or HTZ (100 μmol/L).

To obtain NO-mediated (nitrergic) responses, mouse CC were pretreated with guanethidine (30 μmol/L) and atropine (1 μmol/L) to remove the effects of adrenergic and cholinergic neurons, respectively, and then contracted with U46619 (100 nmol/L). After stable contraction levels were attained, a series of EFS-induced relaxations were constructed (1−32 Hz) in the absence and presence of HTZ (100 μmol/L).[21,25]

## Long-Term Treatments

Long-term treatments were carried out by adding drugs to the drinking water. HTZ was administered at a dose of 50 mg/L to provide a daily intake of approximately 6 mg/kg for 4 weeks. This dose was chosen after testing several dose regimens and aiming for a mean plasma concentration similar to the maximum concentration achieved after a single oral dose of HTZ 25 mg in humans. Amiloride was added to achieve a daily dosage of 0.6 mg/kg.

## HTZ Plasma Determination by Tandem Mass Spectrometry

Animals were anesthetized with isoflurane inhalation (5%) and 1 mL of blood was collected by abdominal aorta puncture. Plasma was obtained by centrifugation of blood samples at 2,000 × *g* for 10 minutes and kept frozen at −20°C until quantification. Plasma HTZ concentration was determined by high-performance liquid chromatography coupled to tandem mass spectrometry using chlortalidone as the internal standard.[32] The mass spectrometer (Quattro Micro; Waters Micromass, Milford, MA, USA), equipped with an electrospray source running in negative mode ($ES^-$), was set up in multiple reaction monitoring of transitions from 295.8 to 77.7 for HTZ.

Data were acquired by MassLynx 4.0 SP4 (Waters Micromass).

## Reverse Transcriptase Polymerase Chain Reaction Analysis

Isolation and purification of CC and kidney total RNA were performed with TRIzol reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). RNA concentration and quality were determined spectrophotometrically and by electrophoresis in 1.2% non-denaturing agarose gels. Synthesis of cDNA was performed using M-MLV reverse transcriptase (200 U/μL; Promega, Madison, WI, USA). A negative control (a reaction that did not contain the RT enzyme) was carried out in parallel with each retro-transcription. Polymerase chain reaction (PCR) amplifications were carried out in 50 μL of final volume using 1 to 2 μL of cDNA as a template. The primers used for this experiment are listed in Table S1. The gene HPRT, which encodes the enzyme hypoxanthine-guanosine phosphoribosyltransferase, was chosen as the endogenous standard for RT-PCR. Amplifications were performed using AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA, USA). PCR products were separated by electrophoresis in 1% (w/v) agarose gels and visualized under ultraviolet light after ethidium bromide staining.

## Total ROCK Activity

Corpora cavernosa from untreated animals were homogenized in a buffer solution containing Tris-HCl 20 mmol/L (pH = 7.5), NaCl 150 mmol/L, ethylenediaminetetraacetic acid 1 mmol/L, ethylene glycol tetraacetic acid 1 mmol/L, Triton X-100 1%, and a protease cocktail inhibitor diluted 1:2,500 (P8340; Sigma, St Louis, MO, USA). Then, homogenates were centrifuged at 12,000 × *g* at 4°C for 10 minutes and the supernatant was used in the assays. Each reaction contained total protein 30 μg and adenosine triphosphate 0.1 mmol/L. Total ROCK activity was evaluated with the CycLex Rho-Kinase Assay Kit (MBL International, Nagoya, Japan) according to the manufacturer's instructions. To evaluate the direct effects of HTZ and amiloride on ROCK activity, the reaction was allowed to occur for 30 minutes in the presence of HTZ (100 μmol/L), amiloride (100 μmol/L), HTZ plus amiloride (both 100 μmol/L), or ROCK inhibitor Y-27632 (10 μmol/L). The effect of long-term treatment on ROCK activity was assessed using CC from control and regularly treated mice. The experimental procedure was performed similarly to that previously described, except that the reaction medium did not contain any diuretic or inhibitor.

## Quantitative PCR Analyses

Each biological sample used in the quantitative PCR experiments corresponded to a pool of CC from three to four mice. Total RNA was extracted from frozen CC of control and regularly treated mice using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA concentration and purity were determined by a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA), and RNA integrity was checked by electrophoresis in 1.2% non-denaturing agarose gels.

Total RNA (1.5 μg) was checked for DNA contamination by conventional PCR and then retro-transcribed with Superscript III (200 U/μL; Invitrogen, Carlsbad, CA, USA) using 100 pmol of random hexamers for priming. Subsequent amplifications were performed in a StepOne Plus real-time detection system (Applied Biosystems) using the SYBR Green PCR master mix (Applied Biosystems). The following genes were analyzed: Rock1 (which encodes ROCK1), Rock2 (ROCK2), Nos1 (nitrergic nitric oxide synthase), Nos3 (endothelial nitric oxide synthase), Gucy1a3 (soluble guanylate cyclase), Pde5a (phosphodiesterase type 5), and Prkg1 (protein kinase G). The constitutive HPRT gene was used as the endogenous standard. Primers used in this work are listed in Table S1.

## Materials

ACh, amiloride, atropine, HTZ, indapamide, indomethacin, L-NAME, phenylephrine, U46619, and guanethidine were obtained from Sigma. U46619, ACh, amiloride, atropine,

guanethidine, KCl, L-NAME, and phenylephrine were dissolved in water, and the other drugs were dissolved in ethanol. The CycLex Rho-Kinase Assay Kit was used.

### Statistical Analysis

All data are expressed as mean ± standard error mean. Instat (GraphPad) was used for statistical analysis. Analysis of variance for repeated measurements was performed for the results and the Bonferroni method was chosen as the post-test. Student unpaired t-tests were used when appropriate. A $P$ value less than .05 was considered significant. $EC_{50}$ values are presented as their negative logarithm ($pEC_{50}$).

## MAIN OUTCOME MEASURES

The main outcome measurement of this study was the generation of stimulation-response curves to assess whether thiazide diuretics had any effect on the contractility of mouse CC. Once observed that this class of diuretics, especially HTZ, had a dramatic impact on isolated CC contractility, the involvement of NO and ROCK pathways was also investigated.

## RESULTS

### Effects of HTZ on Contractions to Phenylephrine

The cumulative addition of phenylephrine (10 nmol/L−10 μmol/L) induced concentration-dependent contractions of the mouse CC smooth muscle. HTZ (10−100 μmol/L; n = 4) caused a significant increase of concentration-dependent contractions to phenylephrine compared with those to phenylephrine alone (control group; Figure 1A, Table 1). Indomethacin (5 μmol/L; n = 7) caused a significant rightward shift in the concentration-response curves to phenylephrine compared with the control group (Figure 1B, Table 2) but did not affect the HTZ-induced leftward shift in the concentration-response curves to phenylephrine (Figure 1B, Table 2).

The addition of L-NAME (100 μmol/L, n = 7) caused an augmented maximum response and a leftward shift in phenylephrine-induced contractions (Figure 1C, Table 2). Addition of HTZ (100 μmol/L; n = 7), in the presence of L-NAME failed to change the contractions induced by phenylephrine in CC strips compared with L-NAME alone (Figure 1C, Table 2). The addition of HTZ to the Krebs

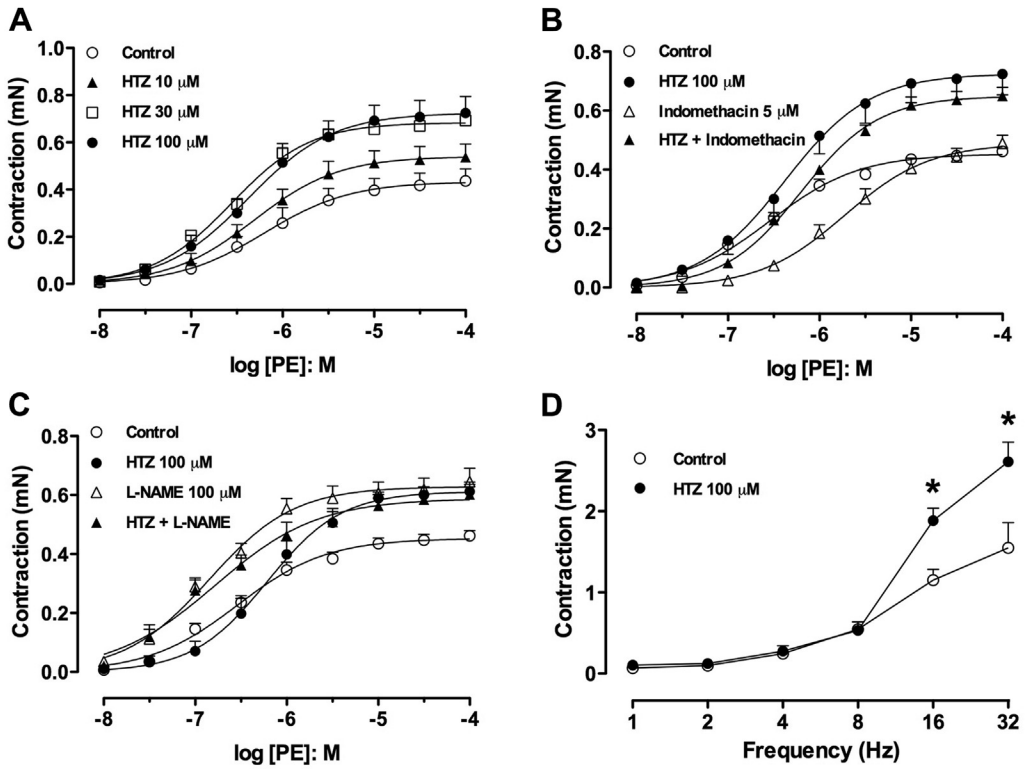


**Figure 1.** Response curves of mouse corpus cavernosum smooth muscle strips to PE and electrical field stimulation. Panel A shows the addition of PE to corpus cavernosum strips alone (control) or after pre-incubation with HTZ (10−100 μmol/L). Panel B shows the addition of PE to corpus cavernosum strips alone or after pre-incubation with HTZ (100 μmol/L), indomethacin (5 μmol/L), or HTZ plus indomethacin. Panel C shows the addition of PE to corpus cavernosum strips alone or after pre-incubation with HTZ (100 μmol/L), L-NAME (100 μmol/L), or HTZ plus L-NAME. Panel D shows contractile responses of mouse corpus cavernosum to electrical field stimulation (1−32 Hz) in the absence (control) or presence of HTZ (100 μmol/L). PE was used at concentrations of 10 nmol/L to 100 μmol/L. Contraction values were calculated by subtracting the basal tone from maximal contractions (milli-newtons) produced by PE or electrical field stimulation. Data are presented as mean ± standard error of the mean of four to eight experiments. *$P$ < .05 vs control for each frequency evaluated. HTZ = hydrochlorothiazide; L-NAME = N(ω)-nitro-L-arginine methyl ester; PE = phenylephrine.

**Table 1.** $pEC_{50}$ and $E_{max}$ obtained from concentration-response curves to phenylephrine (10 nmol/L–100 μmol/L) with and without diuretics in mouse corpus cavernosum smooth muscle*

| | Phenylephrine | | |
|---|---|---|---|
| | $pEC_{50}$ | $E_{max}$ (mN) | Experiments, n |
| Control | 6.20 ± 0.15 | 0.44 ± 0.05 | 8 |
| HTZ 10 μmol/L | 6.31 ± 0.11 | 0.54 ± 0.06 | 6 |
| HTZ 30 μmol/L | 6.56 ± 0.04 | 0.69 ± 0.02† | 4 |
| HTZ 100 μmol/L | 6.37 ± 0.11 | 0.72 ± 0.07‡ | 7 |
| Control | 6.04 ± 0.03 | 0.44 ± 0.05 | 7 |
| Amiloride 100 μmol/L | 5.46 ± 0.07‡ | 0.44 ± 0.05 | 4 |
| Control | 5.54 ± 0.16 | 0.53 ± 0.04 | 5 |
| HTZ 100 μmol/L | 5.27 ± 0.16 | 0.70 ± 0.05† | 8 |
| HTZ 100 μmol/L + amiloride 100 μmol/L | 4.94 ± 0.12† | 0.53 ± 0.04‖ | 13 |
| Control | 6.33 ± 0.10 | 0.48 ± 0.06 | 10 |
| Long-term HTZ (6 mg/kg/d) | 6.38 ± 0.11 | 0.77 ± 0.10† | 4 |
| Control | 6.10 ± 0.15 | 0.47 ± 0.07 | 5 |
| Long-term HTZ + amiloride | 6.14 ± 0.17 | 0.53 ± 0.07 | 5 |

$E_{max}$ = maximal response; HTZ = hydrochlorothiazide; $pEC_{50}$ = potency at half-maximal effective concentration.
*Data presented as mean ± standard error of the mean of the number of experiments. $pEC_{50}$ is represented as the negative logarithm of molar concentration to produce 50% of maximal contractile response elicited by the agonist relative to phenylephrine-induced contractions. $E_{max}$ is calculated as milli-newtons of contraction relative to the maximal changes from the contractile response produced by phenylephrine.
†$P < .05$ vs control group; ‡$P < .01$ vs control group; ‖$P < .05$ vs HTZ group.

solution before phenylephrine did not result in changes to the basal tone.

## Effects of HTZ on Relaxation to ACh

In contrast to its clear modulation of contractions, HTZ did not affect relaxations induced by ACh on CC strips pre-contracted with U46619 (100 nmol/L; $EC_{50}$ = 7.26 ± 0.29 and 7.97 ± 0.29 for control and HTZ, respectively; n = 5).

## Effects of HTZ on Responses to EFS

Under control conditions, the CC strips responded to EFS with frequency-dependent contractions. Exposure of CC strips to HTZ (100 μmol/L, n = 7) for 30 minutes before and during EFS increased the contractile responses (Figure 1D). L-NAME (100 μmol/L, n = 7) increased the contraction to EFS and HTZ could no longer potentiate these responses (as observed with phenylephrine-induced contractions).

In CC strips pre-contracted with U46619 (100 nmol/L; n = 7; 0.39 ± 0.04 and 0.37 ± 0.04 mN, for control and HTZ, respectively), EFS elicited frequency-dependent relaxations that were unaffected by the addition of HTZ (100 μmol/L).

## Effects of Long-Term Treatment In Vivo With HTZ on CC Strips and Response Ex Vivo

The CC strips prepared from mice receiving HTZ (6 mg/kg/day) for 4 weeks showed a significant change in maximal response to phenylephrine and a substantial decrease of EFS-evoked relaxations (Figure 2A and B, Table 1). No effect was observed on relaxations induced by ACh on CC strips pre-contracted with U46619 100 nmol/L ($EC_{50}$ = 7.33 ± 0.13 and 7.38 ± 0.16 for control and treatment, respectively; n = 5).

## HTZ Plasma Concentration on Long-Term Treatment

The HTZ plasma concentration from mice receiving HTZ (6 mg/kg/day) in the drinking water at the end of treatment was 149 ± 56 ng/mL (500 ± 180 nmol/L; n = 6).

## Amiloride Attenuation of HTZ-Induced Potentiation of CC Contractility In Vitro

Amiloride (100 μmol/L, n = 4) significantly attenuated the phenylephrine-induced CC contractions compared with the corresponding control group (Figure 3A, Table 1).

**Table 2.** $pEC_{50}$ and $E_{max}$ obtained from concentration-response curves to phenylephrine (10 nmol/L–100 μmol/L) with and without HTZ (100 μmol/L), L-NAME, and indomethacin in mouse cavernosal smooth muscle*

| | Phenylephrine | | |
|---|---|---|---|
| | $pEC_{50}$ | $E_{max}$ (mN) | Experiments, n |
| Control | 6.24 ± 0.14 | 0.46 ± 0.02 | 7 |
| L-NAME 100 μmol/L | 6.89 ± 0.14‡ | 0.65 ± 0.05† | 7 |
| HTZ 100 μmol/L | 6.20 ± 0.13 | 0.61 ± 0.02† | 7 |
| HTZ 100 μmol/L + L-NAME 100 μmol/L | 6.79 ± 0.16† | 0.60 ± 0.04† | 7 |
| Control | 6.25 ± 0.14 | 0.46 ± 0.02 | 5 |
| Indomethacin 5 μmol/L | 5.69 ± 0.11† | 0.48 ± 0.02 | 7 |
| HTZ 100 μmol/L | 6.37 ± 0.11 | 0.70 ± 0.02† | 7 |
| HTZ 100 μmol/L + indomethacin 5 μmol/L | 6.19 ± 0.05§ | 0.60 ± 0.05†,§ | 7 |

$E_{max}$ = maximal response; HTZ = hydrochlorothiazide; L-NAME = N(ω)-nitro-L-arginine methyl ester; $pEC_{50}$ = potency at half-maximal effective concentration.
*Data presented as mean ± standard error of the mean of the number of experiments. $pEC_{50}$ is represented as the negative logarithm of molar concentration to produce 50% of maximal contractile response elicited by an agonist relative to phenylephrine-induced contractions. $E_{max}$ is calculated as milli-newtons of contraction relative to the maximal changes from the contractile response produced by phenylephrine.
†$P < .05$ vs control group; ‡$P < .01$; §$P < .05$ vs indomethacin group.

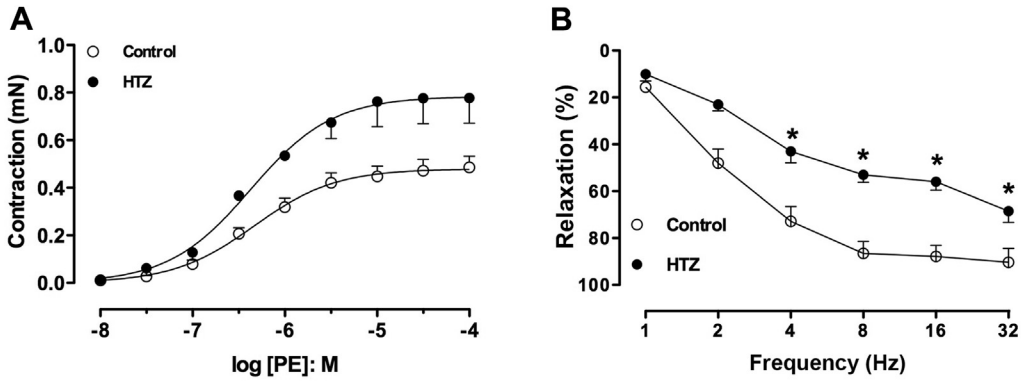


**Figure 2.** Concentration-response curves of mouse corpus cavernosum smooth muscle strips to PE and relaxant response to electrical field stimulation after long-term administration of HTZ in vivo. Panel A shows the addition of PE to corpus cavernosum strips from untreated (control) and HTZ-treated mice. Panel B shows the relaxant responses to electrical field stimulation (1–32 Hz) in untreated (control) and HTZ-treated animals. *$P < .05$ vs control for each frequency evaluated. PE was used at concentrations of 10 nmol/L to 100 μmol/L. Contraction values were calculated by subtracting the basal tone from maximal contractions (milli-newtons) produced by PE or electrical field stimulation. Data are presented as mean ± standard error of the mean of 4 to 10 experiments. HTZ = hydrochlorothiazide; PE = phenylephrine.

Pre-incubation of CC strips with amiloride plus HTZ (100 μmol/L for both; n = 13) reversed the increase in maximal response observed with HTZ alone (100 μmol/L; n = 8) compared with the control. The rightward shift previously observed with amiloride alone was maintained even in the presence of HTZ (Figure 3B, Table 1).

## Effects of Long-Term Treatment In Vivo With HTZ Plus Amiloride on CC Strips and Response In Vitro

Changes in maximal response to phenylephrine and reduction of EFS-evoked relaxations were not observed in CC strips prepared from mice receiving HTZ plus amiloride (6 mg/kg/day and 0.6 mg/kg/day, respectively) over 4 weeks (Figure 4A and B, Table 2). Moreover, regular treatment did not affect Ach-induced relaxations of CC strips pre-contracted with U46619 ($EC_{50}$ 7.7 ± 0.11 and 7.8 ± 0.2 for control and treatment respectively, n = 5).

## NaCl Cotransporter and ENaC Expression

Transcription of the Scnn1a gene, which encodes the α subunit of the ENaC, a known target of amiloride, was readily detected in mouse CC. However, Slc12a3 gene transcripts, encoding the thiazide sensitive NaCl cotransporter, could not be amplified (n = 2; Figure 5).

## Diuretic-Induced Changes in ROCK Activity

Amiloride inhibited ROCK activity in vitro alone (82 ± 3.7% of control activity; n = 6; $P < .01$; Figure 6A) or with HTZ (78 ± 3.8% of control activity; n = 6; $P < .001$). After incubation with ROCK inhibitor Y-27632, total activity was decreased to 28 ± 1.5%. Treatment of mice for 4 weeks with HTZ plus amiloride decreased total ROCK activity ex vivo (76 ± 5.3%; Figure 6B) compared with the control group (n = 7; $P < .01$). The ROCK activation assay did not detect any influence of HTZ on ROCK

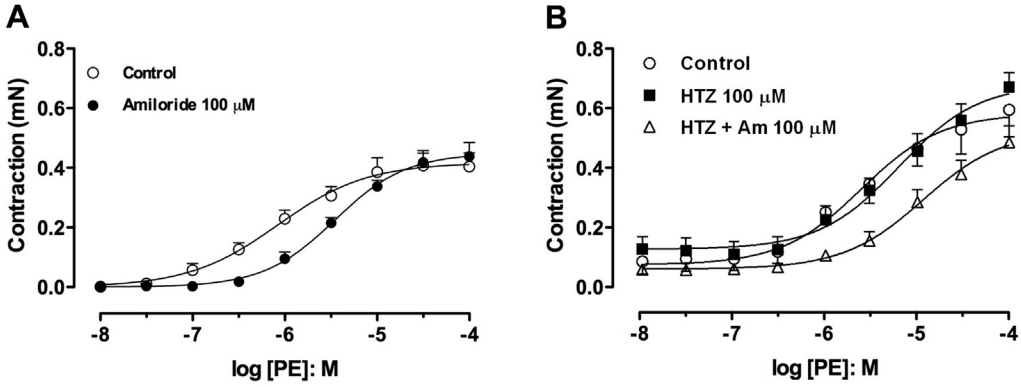


**Figure 3.** Concentration-response curves of mouse corpus cavernosum smooth muscle strips to PE in the presence of Am. Panel A shows the addition of PE to corpus cavernosum strips alone (control) and after pre-incubation with Am (100 μmol/L). Panel B shows the addition of PE to corpus cavernosum strips alone and after pre-incubation with HTZ (100 μmol/L) or HTZ + Am. PE was used at concentrations of 10 nmol/L to 100 μmol/L. Contraction values were calculated by subtracting the basal tone from maximal contractions (milli-newtons) produced by PE. Data are presented as mean ± standard error of the mean of 4 to 13 experiments. Am = amiloride; HTZ = hydrochlorothiazide; PE = phenylephrine.

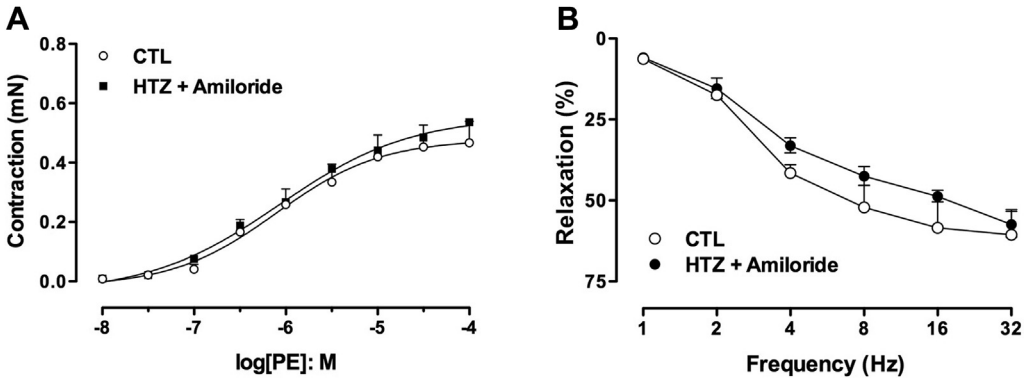


**Figure 4.** Concentration-response curves of mouse corpus cavernosum smooth muscle strips to PE and relaxant response to electrical field stimulation after long-term coadministration of HTZ and amiloride in vivo. Panel A shows the addition of PE to corpus cavernosum strips from untreated mice (control) and mice treated with HTZ plus amiloride. Panel B shows relaxant responses to electrical field stimulation (1–32 Hz) in untreated mice (control) and mice treated with HTZ plus amiloride. PE was used at concentrations of 10 nmol/L to 100 μmol/L. Contraction values were calculated by subtracting the basal tone from maximal contractions (milli-newtons) produced by PE or electrical field stimulation. Data are presented as mean ± standard error of the mean of five experiments. CTL = control; HTZ = hydrochlorothiazide; PE = phenylephrine.

activity acutely in vitro (92 ± 3.7% of control activity; n = 6; $P > .05$; Figure 6A) or ex vivo after long-term treatment (89 ± 7.0% of control activity; n = 7; $P > .05$; Figure 6B).

## Quantitative RT-PCR

Quantitative real-time PCR was used to analyze any possible effect of HTZ or HTZ plus amiloride treatment on the expression of genes encoding proteins involved in penile erection. No alteration in Rock1, Rock2, Nos1, Nos3, Gucy1a3, or Pkrg1 transcripts levels was observed in HTZ-treated mice compared with the control group, whereas a 20% decrease was observed for Pde5a transcripts. However, long-term treatment with amiloride plus HTZ produced 40% and 55% downregulation of Rock1 and Nos1 gene expression, respectively (Table 3).

## DISCUSSION

Our results have clearly demonstrated that HTZ potentiates phenylephrine-induced and EFS-induced contractions of mouse CC strips in vitro. This effect also was observed ex vivo after long-term treatment with HTZ. This action appeared to be specific to thiazide-like diuretics, because the same effect was obtained with indapamide (Figure S1) but was not observed with the potassium-sparing diuretic amiloride. This effect is not related to the known target of HTZ, the NaCl cotransporter (Slc12A3 gene), because RT-PCR did not detect its expression in mouse CC. This is the first report of HTZ-induced potentiation of CC contractility, which could explain the association of ED with thiazide-based therapies. This conclusion, although robust, might be further strengthened by an in vivo assessment of erectile function after HTZ treatment that additional studies should address. Smooth muscle tone and agonist-elicited responses are influenced by levels of endogenous cyclooxygenase products.[33–35] Inhibition of cyclooxygenases by diclofenac (50 mg) or rofecoxib (25 mg) significantly decreases the diuretic-natriuretic effect of HTZ in humans, indicating a possible role for prostaglandins in the renal effects of thiazides.[36] Indomethacin potentiated ACh-induced relaxation of the rabbit CC, indicating the presence of contractor cyclooxygenase products.[37] This would be compatible with our results in which indomethacin caused a rightward shift of phenylephrine-induced contractions, strongly suggesting the loss of a contractor cyclooxygenase product. However, HTZ could still potentiate phenylephrine-induced contractions of indomethacin-treated CC strips, ruling out a mechanism based on cyclooxygenase products as an explanation of HTZ-induced potentiation.

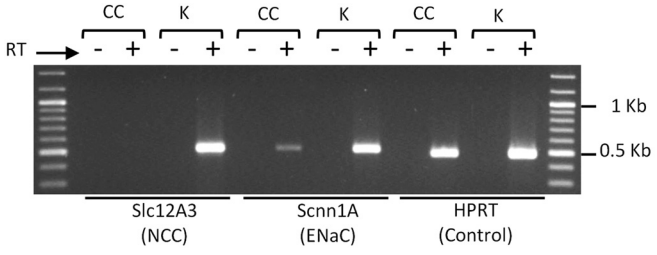


**Figure 5.** RT polymerase chain reaction with cDNA from mouse tissues (n = 2). Reactions were produced with (+) and without (−) RT. The genes (corresponding encoded proteins) under study appear below the respective gels. CC = corpus cavernosum; ENaC = epithelial sodium channel; K = kidney; NCC = NaCl cotransporter; RT = reverse transcriptase.

Inhibition of NO biosynthesis increases contractile responses in many smooth muscle preparations.[38–40] However, thiazide diuretics did not potentiate ACh-induced relaxations of rat aorta, indicating that their action on blood vessels can be independent of NO release.[41] Interestingly, in our experiments, HTZ failed to potentiate phenylephrine-induced contractions in the presence of the NO synthesis inhibitor L-NAME in vitro, suggesting an

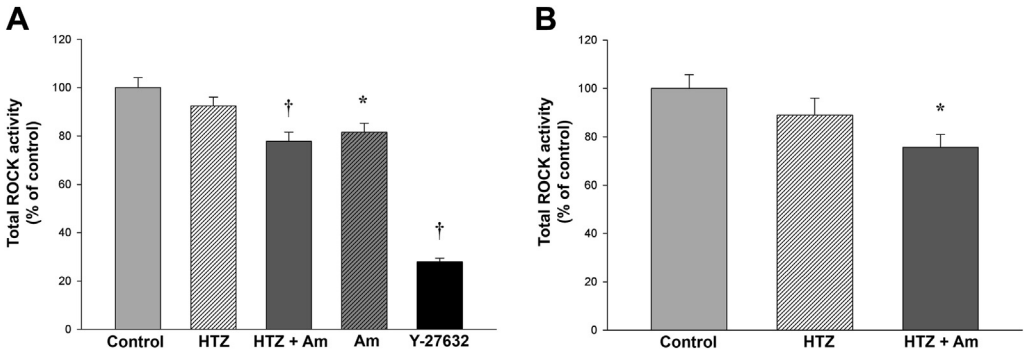


**Figure 6.** Evaluation of ROCK activity. Panel A shows the influence of HTZ (100 $\mu$mol/L), Am (100 $\mu$mol/L), HTZ + Am, and Y-27632 on ROCK activity, expressed as the mean percentage of control ± standard error of the mean. Control = 100 ± 4.0; HTZ = 92 ± 3.7; HTZ + Am = 78 ± 3.8; Am = 82 ± 3.7; Y-27632 = 28 ± 1.5. *$P < .01$ vs control group; †$P < .001$ vs control group; n = 6 for all groups. Panel B shows total ROCK activity, expressed as mean percentage ± standard error of the mean compared with the control group. Control = 100 ± 5.7%; HTZ = 89 ± 7.0%; HTZ + Am = 76 ± 5.3%. *$P < .01$ vs control group; n = 7 for all groups. Am = amiloride; HTZ = hydrochlorothiazide; ROCK = Rho-kinase.

involvement of the NO pathway in this potentiation. However, HTZ affected neither the relaxation induced by ACh nor that induced by EFS in mouse CC in vitro, ruling out NO involvement in its action. One possible explanation is that the leftward shift caused by NO inhibition "overrides" other mechanisms, because of the major role of NO as the relaxation agent in this tissue.

After regular treatment with HTZ for 4 weeks, potentiation of phenylephrine-induced contraction was observed ex vivo, similarly to the in vitro response to the addition of HTZ, indicating an adaptive response of CC to HTZ. The CC contractile response to EFS was similar to the control after long-term treatment with HTZ, diverging from the different responses observed in vitro. These changes in EFS response patterns after long-term treatment could result from adaptations that might compensate one another. An increase of adrenergic sensitivity as a whole could explain this result. In fact, when using phenylephrine only, the $\alpha_1$-adrenergic response is observed. In contrast, when EFS stimuli are used, $\alpha_1$- and $\beta_3$-adrenoceptors are stimulated simultaneously. The $\beta_3$-adrenoceptors relax human CC in vitro by inhibition of the ROCK pathway[42]; thus, the increase in sensitivity of these two antagonistic pathways would result in an unchanged contractile response. However, this explanation is not supported by our results, because we did not find a significant diminution of ROCK activity after long-term treatment with HTZ.

**Table 3.** Abundance of transcripts of Rock1, Rock2, Nos1, Nos3, Gucy1a3, Pde5a, and Prkg1 in mouse corpora cavernosa*

| | Fold change in gene expression | | |
|---|---|---|---|
| Gene name | Control group | HTZ group | HTZ + Am group |
| Rock1 | 1.01 ± 0.11 | 1.11 ± 0.15 | 0.61 ± 0.06† |
| Rock2 | 1.02 ± 0.15 | 0.91 ± 0.29 | 1.0 ± 0.19 |
| Nos1 | 1.0 ± 0.09 | 0.8 ± 0.19 | 0.45 ± 0.1† |
| Nos3 | 1.0 ± 0.05 | 1.08 ± 0.13 | 0.87 ± 0.06 |
| Gucy1a3 | 1.0 ± 0.08 | 0.97 ± 0.23 | 0.93 ± 0.16 |
| Pde5a | 1.0 ± 0.04 | 0.79 ± 0.01† | 0.98 ± 0.12 |
| Prgk1 | 1.0 ± 0.05 | 0.91 ± 0.06 | 0.99 ± 0.22 |

Control = untreated mice; HTZ = mice treated with hydrochlorothiazide; HTZ + Am = mice treated with hydrochlorothiazide plus amiloride.
*Messenger RNA levels were analyzed by quantitative real-time polymerase chain reaction and normalized in relation to those of the HPRT gene. Average values ± standard error of the mean of three independent biological replicas are represented.
†$P < .05$ vs control group.

Thiazide diuretics attenuate agonist-induced vasoconstriction in vivo and in vitro by calcium desensitization of vascular smooth muscle cells, which is linked to the RhoA-Rho kinase pathway.[43] The expression of RhoA in rabbit CC is 17-fold higher that in ileum smooth muscle, and inhibition or stimulation of this pathway directly affects erectile function.[44,45] In our experiments, HTZ failed to potentiate total ROCK activity in vitro and long-term treatment with HTZ did not influence the expression of Rock1 and Rock2 or total ROCK activity. Further, stimulation of the ROCK pathway decreases endothelial NO synthase activity and expression,[45,46] which was not observed in this study, corroborating the unchanged response to ACh-induced relaxation observed in the ex vivo experiments.

Long-term HTZ treatment resulted in decreased EFS-induced relaxation diverging from the similar relaxation observed in vitro. This decrease cannot be explained by modifications in the expression of Nos1, Gucy1a3, or Prkg1, because the transcription level of these genes was unaltered in the HTZ group.

These results could be clinically important, because ED has been reported after in vivo treatment with thiazides.[13–18] From the preceding discussion, it is likely that contraction of the non-vascular smooth muscle of the CC could compromise the effects of any vasodilatation and thus weaken the erectile response. The concentration of HTZ achieved therapeutically, approximately 130 ng/mL after ingesting a 25-mg tablet, is close to that found to potentiate ex vivo the phenylephrine-induced contractions of mouse CC.[32] Long-term exposure to this concentration in vivo resulted in an increase of approximately 50% of the maximal

response to phenylephrine ex vivo. It is relevant to note that the ED caused by HTZ is more evident with higher doses of the diuretic.[15,18]

Transcripts of the main target of amiloride, the ENaC, were detected in mouse CC by RT-PCR. This finding is interesting because amiloride caused an attenuation of phenylephrine-induced contraction, even in the presence of HTZ. In vascular smooth muscle, ENaC is considered a mediator in the regulation of pressure-induced vasoconstriction.[47,48] Usually, amiloride is not used clinically as a single agent; therefore, there are no clear data available on their effects on erectile function. In the present work, amiloride inhibited total ROCK activity in vitro and ex vivo. This inhibition could explain why amiloride reversed the HTZ-induced potentiation of CC contractility to phenylephrine. The precise molecular mechanism by which amiloride exerts this effect is not fully understood. However, our results show a downregulation of Rock1 gene expression after long-term amiloride plus HTZ treatment, suggesting the involvement of an unidentified transcriptional regulator.

Consistent with the attenuation of phenylephrine-induced contraction, long-term treatment with amiloride plus HTZ also reversed the decreased EFS-induced relaxation observed with HTZ alone. This observation is surprising considering the twofold downregulation of Nos1 gene expression found in the HTZ plus amiloride group. However, despite the unchanged Pde5a expression levels, it cannot be ruled out that a counteracting decrease in phosphodiesterase type 5 activity occurs.

Overall, these observations suggest that coadministration of amiloride in patients who develop HTZ-induced ED might ameliorate this adverse effect. However, more studies are needed to support this hypothesis.

## CONCLUSION

In this work, we clearly demonstrate that HTZ in vitro and after exposure in vivo with concentrations achieved therapeutically potentiates CC smooth muscle contraction. This effect is not mediated by the NaCl cotransporter or by activation of the ROCK pathway. An increased adrenergic response or sensitivity and decreased NO-mediated relaxation could play a major role in this phenomenon. However, the elucidation of the precise mechanisms requires further investigation. These findings could explain why HTZ is associated with ED in humans.

**Corresponding Author:** Mauro Napolitano, PhD, Department of Pharmacology, Institute of Biomedical Sciences, Avda Professor Lineu Prestes, 1524, CEP 05508-000, São Paulo, SP, Brazil. Tel: +55-11-3091-7493; E-mail: mauronapolitano@gmail.com

*Conflict of Interest:* The authors report no conflicts of interest.

*Funding:* None.

## STATEMENT OF AUTHORSHIP

### Category 1

(a) **Conception and Design**
Thiago Gagliano-Jucá; Mauro Napolitano; Edson Antunes; Anibal Gil Lopes; Gilberto De Nucci

(b) **Acquisition of Data**
Thiago Gagliano-Jucá; Mauro Napolitano; Fernanda Del Grossi Ferraz Carvalho; Rafael Campos; Fabíola Zakia Mónica; Mário Angelo Claudino

(c) **Analysis and Interpretation of Data**
Thiago Gagliano-Jucá; Mauro Napolitano; Fernanda Del Grossi Ferraz Carvalho; Rafael Campos; Fabíola Zakia Mónica; Mário Angelo Claudino; Edson Antunes; Anibal Gil Lopes; Gilberto De Nucci

### Category 2

(a) **Drafting the Article**
Thiago Gagliano-Jucá; Mauro Napolitano

(b) **Revising It for Intellectual Content**
Thiago Gagliano-Jucá; Mauro Napolitano; Gilberto De Nucci

### Category 3

(a) **Final Approval of the Completed Article**
Thiago Gagliano-Jucá; Mauro Napolitano; Gilberto De Nucci

## REFERENCES

1. Conway J, Lauwers P. Hemodynamic and hypotensive effects of long-term therapy with chlorothiazide. **Circulation 1960; 21:21-27.**
2. van Brummelen P, Man in 't Veld AJ, Schalekamp MA. Hemodynamic changes during long-term thiazide treatment of essential hypertension in responders and nonresponders. **Clin Pharmacol Ther 1980;27:328-336.**
3. Vardan S, Mookherjee S, Warner R, et al. Systolic hypertension in the elderly. Hemodynamic response to long-term thiazide diuretic therapy and its side effects. **JAMA 1983;250:2807-2813.**
4. Preziosi P, De Schaepdryver AF, Marmo E, et al. On the mechanism of the anti-hypertensive effect of hydrochlorothiazide. **Arch Int Pharmacodyn Ther 1961;131:209-229.**
5. Gillenwater JY, Scott JB, Frohlich ED. Effect of chlorothiazide on response of renal vascular bed to vasoactive substances. **Circ Res 1962;11:283-286.**
6. Calder JA, Schachter M, Sever PS. Direct vascular actions of hydrochlorothiazide and indapamide in isolated small vessels. **Eur J Pharmacol 1992;220:19-26.**
7. Calder JA, Schachter M, Sever PS. Ion channel involvement in the acute vascular effects of thiazide diuretics and related compounds. **J Pharmacol Exp Ther 1993;265:1175-1180.**
8. Pickkers P, Hughes AD, Russel FG, et al. Thiazide-induced vasodilation in humans is mediated by potassium channel activation. **Hypertension 1998;32:1071-1076.**
9. Simon DB, Nelson-Williams C, Bia MJ, et al. Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. **Nat Genet 1996;12:24-30.**

10. Zsoter TT, Suffiad K. Effect of hydrochlorothiazide on calcium kinetics in blood vessels. **Can J Physiol Pharmacol 1973; 51:579-582.**
11. Moura AM, Worcel M. Mode of action of cyclothiazide and triamterene. Ex vivo effect on 22Na and 86Rb efflux from arterial smooth muscle. **Eur J Pharmacol 1982;86:129-133.**
12. Pickkers P, Hughes AD. Relaxation and decrease in [Ca2+]i by hydrochlorothiazide in guinea-pig isolated mesenteric arteries. **Br J Pharmacol 1995;114:703-707.**
13. Chang SW, Fine R, Siegel D, et al. The impact of diuretic therapy on reported sexual function. **Arch Intern Med 1991; 151:2402-2408.**
14. Wassertheil-Smoller S, Blaufox MD, Oberman A, et al. Effect of antihypertensives on sexual function and quality of life: the TAIM Study. **Ann Intern Med 1991;114:613-620.**
15. Prisant LM, Carr AA, Bottini PB, et al. Sexual dysfunction with antihypertensive drugs. **Arch Intern Med 1994;154:730-736.**
16. Rosen RC. Sexual dysfunction as an obstacle to compliance with antihypertensive therapy. **Blood Press Suppl 1997; 1:47-51.**
17. Grimm RH Jr, Grandits GA, Prineas RJ, et al. Long-term effects on sexual function of five antihypertensive drugs and nutritional hygienic treatment in hypertensive men and women. Treatment of Mild Hypertension Study (TOMHS). **Hypertension 1997;29:8-14.**
18. Kloner R. Erectile dysfunction and hypertension. **Int J Impot Res 2007;19:296-302.**
19. Chrysant SG. Antihypertensive therapy causes erectile dysfunction. **Curr Opin Cardiol 2015;30:383-390.**
20. Lue TF. Erectile dysfunction. **N Engl J Med 2000; 342:1802-1813.**
21. Saenz de Tejada I, Goldstein I, Krane RJ. Local control of penile erection. Nerves, smooth muscle, and endothelium. **Urol Clin North Am 1988;15:9-15.**
22. Andersson KE, Wagner G. Physiology of penile erection. **Physiol Rev 1995;75:191-236.**
23. Rodenburg EM, Visser LE, Hoorn EJ, et al. Thiazides and the risk of hypokalemia in the general population. **J Hypertens 2014;32:2092-2097.**
24. Blanchard A, Vargas-Poussou R, Vallet M, et al. Indomethacin, amiloride, or eplerenone for treating hypokalemia in Gitelman syndrome. **J Am Soc Nephrol 2015;26:468-475.**
25. Teixeira CE, Teixeira SA, Antunes E, et al. The role of nitric oxide on the relaxations of rabbit corpus cavernosum induced by Androctonus australis and Buthotus judaicus scorpion venoms. **Toxicon 2001;39:633-639.**
26. Teixeira CE, Baracat JS, Zanesco A, et al. Atypical beta-adrenoceptor subtypes mediate relaxations of rabbit corpus cavernosum. **J Pharmacol Exp Ther 2004;309:587-593.**
27. Teixeira CE, Priviero FB, Webb RC. Effects of 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4-ylamine (BAY 41-2272) on smooth muscle tone, soluble guanylyl cyclase activity, and NADPH oxidase activity/expression in corpus cavernosum from wild-type, neuronal, and endothelial nitric-oxide synthase null mice. **J Pharmacol Exp Ther 2007;322:1093-1102.**
28. Eckenfels A, Vane JR. Prostaglandins, oxygen tension and smooth muscle tone. **Br J Pharmacol 1972;45:451-462.**
29. Rand MJ, Li CG. The inhibition of nitric oxide-mediated relaxations in rat aorta and anococcygeus muscle by diphenylene iodonium. **Clin Exp Pharmacol Physiol 1993;20:141-148.**
30. Dalaklioglu S, Ozbey G. The potent relaxant effect of resveratrol in rat corpus cavernosum and its underlying mechanisms. **Int J Impot Res 2013;25:188-193.**
31. Hennenberg M, Miljak M, Herrmann D, et al. The receptor antagonist picotamide inhibits adrenergic and thromboxane-induced contraction of hyperplastic human prostate smooth muscle. **Am J Physiol Renal Physiol 2013;305:F1383-F1390.**
32. Patel RB, Patel UR, Rogge MC, et al. Bioavailability of hydrochlorothiazide from tablets and suspensions. **J Pharm Sci 1984;73:359-361.**
33. Robinson D, Khullar V, Cardozo L. Pharmacological management of detrusor instability. **Int Urogynecol J Pelvic Floor Dysfunct 2001;12:271-278.**
34. Feletou M, Huang Y, Vanhoutte PM. Vasoconstrictor prostanoids. **Pflugers Arch 2010;459:941-950.**
35. Wong MS, Vanhoutte PM. COX-mediated endothelium-dependent contractions: from the past to recent discoveries. **Acta Pharmacol Sin 2010;31:1095-1102.**
36. Knauf H, Bailey MA, Hasenfuss G, et al. The influence of cardiovascular and antiinflammatory drugs on thiazide-induced hemodynamic and saluretic effects. **Eur J Clin Pharmacol 2006;62:885-892.**
37. Minhas S, Cartledge JJ, Eardley I, et al. The interaction of nitric oxide and prostaglandins in the control of corporal smooth muscle tone: evidence for production of a cyclooxygenase-derived endothelium-contracting factor. **BJU Int 2001;87:882-888.**
38. Jeremy JY, Mikhailidis DP. Cigarette smoking and erectile dysfunction. **J R Soc Promot Health 1998;118:151-155.**
39. Azadzoi KM, Saenz de Tejada I. Hypercholesterolemia impairs endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. **J Urol 1991;146:238-240.**
40. Ignarro LJ. Endothelium-derived nitric oxide: actions and properties. **FASEB J 1989;3:31-36.**
41. Zhou MS, Schulman IH, Jaimes EA, et al. Thiazide diuretics, endothelial function, and vascular oxidative stress. **J Hypertens 2008;26:494-500.**
42. Cirino G, Sorrentino R, di Villa Bianca Rd, et al. Involvement of beta 3-adrenergic receptor activation via cyclic GMP- but not NO-dependent mechanisms in human corpus cavernosum function. **Proc Natl Acad Sci U S A 2003;100:5531-5536.**
43. Zhu Z, Zhu S, Liu D, et al. Thiazide-like diuretics attenuate agonist-induced vasoconstriction by calcium desensitization linked to Rho kinase. **Hypertension 2005;45:233-239.**
44. Chitaley K, Wingard CJ, Clinton Webb R, et al. Antagonism of Rho-kinase stimulates rat penile erection via a nitric oxide-independent pathway. **Nat Med 2001;7:119-122.**

45. Wang H, Eto M, Steers WD, et al. RhoA-mediated Ca2+ sensitization in erectile function. **J Biol Chem 2002; 277:30614-30621.**

46. Sugimoto M, Nakayama M, Goto TM, et al. Rho-kinase phosphorylates eNOS at threonine 495 in endothelial cells. **Biochem Biophys Res Commun 2007;361:462-467.**

47. Jernigan NL, Drummond HA. Vascular ENaC proteins are required for renal myogenic constriction. **Am J Physiol Renal Physiol 2005;289:F891-F901.**

48. Jernigan NL, Drummond HA. Myogenic vasoconstriction in mouse renal interlobar arteries: role of endogenous beta and gammaENaC. **Am J Physiol Renal Physiol 2006;291:F1184-F1191.**

## SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.esxm.2016.02.003.