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Nitrergic relaxations and phenylephrine contractions are not compromised in isolated urethra in a rat model of diabetes

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ABSTRACT

In vivo experiments in a diabetic rat model revealed compromised nitrergic urethral relaxations and increased sensitivity to adrenergic agonists. This study evaluated contractile and relaxation properties of urethral smooth muscle after streptozotocin (STZ)-induced diabetes, in vitro, with the aim of determining whether in vivo deficiencies are related to smooth muscle dysfunction.

Urethral tissue was collected from adult female Sprague–Dawley rats naive, STZ-treated, vehicle-treated and sucrose-fed at 9–12 week post treatment. Strips from proximal, mid, and distal urethra were placed in tissue baths and stimulated using electric field stimulation (EFS) and pharmacological agents. nNOS staining was evaluated using immunohistochemistry.

Phenylephrine (PE, 10 μM) contracted all urethral strips with the highest amplitude in mid urethra, in all treatment groups. Likewise, EFS-induced relaxation amplitudes were larger and were observed more frequently in mid urethra. Relaxations were inhibited by the NOS inhibitor, L-NAME (1–100 μM). Sodium nitroprusside (0.01–1 μM), an NO donor, reversed PE-induced contractions. No statistical differences were observed between treatment groups with respect to any parameters. Qualitative immunohistochemistry showed no differences in the urethral nNOS innervation patterns across the treatment groups.

In summary, nitrergic relaxations and adrenergic-induced contractions in the isolated diabetic rat urethra display similar properties to controls, suggesting no dysfunction on the nitrergic or alpha1 adrenergic receptor function in the smooth muscle. This further implies that compromised urethral relaxation and increased adrenergic agonist sensitivity observed in vivo in this model may be due to the disruption of neural signaling between the urethra and the spinal cord, or within the CNS.

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1. Introduction

Voiding is achieved by simultaneous bladder contraction and urethra relaxation. This is accomplished by the activation of the parasympathetic efferent neurons which release acetylcholine (ACh) to contract the bladder smooth muscle and nitric oxide (NO) to relax the urethral smooth muscle. Simultaneously, the excitatory input to external urethral sphincter (EUS) from spinal motor neurons is inhibited to allow urine flow. The relaxation of the urethra is crucial for efficient voiding and the role of NO in the relaxation has been well established. Anatomical studies using retrograde axonal staining have shown that nitric oxide synthase (NOS) positive fibers in the major pelvic ganglia (MPG) project primarily to the urethra (Vizzard et al., 1994). Functional studies have shown that urethral relaxations induced by EFS in vitro (Persson et al., 1992) and urethral relaxations triggered by bladder contraction in vivo (Bennett et al., 1995) are sensitive to the inhibition of NO synthesis. Furthermore, EFS-induced relaxations are abolished in tissue from rats in which the MPG was removed (Persson et al., 1998), suggesting that NO released from parasympathetic neurons plays a significant role in urethral relaxation.

Storage of urine involves tonic activation of the sympathetic system, which releases norepinephrine (NE). NE acts on beta adrenergic receptors (β-AR) to relax the bladder smooth muscle and on alpha 1 adrenergic receptors (α1-AR) to contract the urethral smooth muscle. In addition, the excitatory motor input to the EUS is turned on to maintain continence, especially when stress is applied to the bladder (de Groat, 2006; Fowler et al., 2008; Thor and de Groat, 2010). α1-AR antagonists have been shown to reduce urethra pressure in vivo and are commonly used in the treatment of the symptoms of lower urinary tract (LUT) obstruction (Andersson and Gratzke, 2007).

Diabetes myelitis, a prevalent and increasingly worldwide disease, greatly affects the LUT function. Patients with diabetes experience

Abbreviations: ACh, Acetylcholine; LUT, Lower Urinary Tract; NO, Nitric Oxide; STZ, Streptozotocin; PE, Phenylephrine; nNOS, neuronal Nitric Oxide Synthesis; SNP, Sodium nitroprusside.

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voiding dysfunctions characterized by increased bladder capacity, poor emptying, elevated post-residual volume and overflow incontinence (Daneshgari et al., 2009; Gomez et al., 2011; Kirschner-Hermanns et al., 2012). The STZ-treated diabetic rat model has been extensively used as a rodent model of the voiding dysfunction encountered in diabetic patients. In this model, there are time-dependent morphological and functional changes in myogenic and neurogenic components of the bladder and urethra that affect both voiding and storage phases (Malmgren et al., 1989; Liu and Daneshgari, 2005; Daneshgari et al., 2006, 2009; Hanna-Mitchell et al., 2013). Studies focused on urethra have shown that the STZ-treated rats exhibited a series of deficits, such as an increase in the intravesical bladder pressure threshold for inducing urethral relaxation (Torimoto et al., 2004), alterations in the pattern, amplitude and frequency of high frequency oscillations of EUS (Torimoto et al., 2004; Yang et al., 2007, 2010; Gu et al., 2012) and occurrence of detrusor sphincter dyssynergia (in approximately 30% of STZ-treated rats) (Yang et al., 2007). Furthermore, urethra relaxations were less sensitive to inhibition by Nω-nitro-L-arginine, which inhibits the NOS enzyme that produces NO, and more sensitive to the alpha-adrenergic agonist, L-phenylephrine (Ueda et al., 1997; Torimoto et al., 2004; Yang et al., 2007, 2010). Together, these changes suggest increased resistance of the urethral outlet in STZ-treated animals.

In this study, we examine whether the reported in vivo STZ-induced deficits in NO and phenylephrine (PE) sensitivity are due to deficits in peripheral urethral smooth muscle contractile properties.

2. Materials and methods

2.1. Animals

This study was conducted in accordance to the protocols established within Urogenix, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility, and approved by the Urogenix Institutional Animal Care and Use Committee which follows the NIH Guidelines for the Care and Use of Laboratory Animals. Female Sprague Dawley rats (200–250 g; ~10–12 weeks old) were obtained from Charles Rivers (Raleigh, NC).

2.2. Induction of diabetes and polyuria

Animals were divided into four groups. One group was injected with streptozotocin (STZ) (*N*-(methylnitrosocarbamoyl)-α-D-glucosamine; Sigma, St. Louis, MO) at 65 mg/kg, i.p. dissolved in 0.02 M sodium citrate buffer (Sigma, St. Louis, MO). A second group of age matched animals received the vehicle for STZ, 0.02 M citrate buffer. The third group was fed 5% sucrose in water to induce polyuria. The fourth group of rats received no treatment, naive. After a period of 7 day post treatment and again on the day of the experiment (9–12 week post treatments), approximately 0.2 μl of blood was collected via lateral tail vein puncture and blood glucose levels were measured using a Precision Xtra system (Abbott Laboratories, Alameda, CA). Successful induction of diabetes was determined if glucose levels were above 250 mg/dl. Approximately 90% of animals in the colony met this criterion. Animals were used at 9–12 week post treatments described above, based on previous studies that showed NO and PE deficiencies in vivo (Torimoto et al., 2004; Yang et al., 2007, 2010).

2.3. In vitro contractility

Rats were deeply anesthetized with isoflurane (4%) and euthanized by exsanguination. The bladder and urethra were removed intact and placed in aerated Krebs solution (composition in mM: NaCl 118.3, $NaHCO_3$ 24.9, KH_2PO_4 1.2, Glucose 11.7, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 1.2). The pH was maintained at 7.4 by oxygenation with 95% O_2/5% CO_2 and osmolarity was between 280 and 310 mOsm. The bladder and urethra were pinned down in a dissecting dish. The bladder was then separated from the urethra 2 mm below the ureterovesicular junction. The urethra, containing both the external striated muscle and the internal smooth muscle layers, was dissected into 3 circular rings of ~2 mm length, termed proximal, mid and distal relative to the bladder neck. Each ring was opened and strips were mounted with the circular smooth muscle oriented longitudinally in double jacketed organ bath (10 ml volume) (Radnoti, Monrovia, CA, and Nihon Kohden, Japan) containing aerated Krebs solution kept at 37 °C. One longitudinal bladder strip was included in each experiment for comparison. Stimulus evoked contractions or relaxations were measured with force displacement transducers (FT03, Radnoti, US, and TB-611T, Nihon Kohden, Japan). Electrical stimulation (0.25 ms square wave pulse; 1–20 Hz; 10–80 V) was applied for 10 s periods every 90 s through field stimulation electrodes (Radnoti, Monrovia, CA and Nihon Kohden, Japan) coupled to a Grass S88 (Grass Instruments, W. Warwick, RI) or a Nihon Kohden, SEN-3401 stimulator (Nihon Kohden, Japan). EFS-induced responses were tetrodotoxin (TTX; 0.5 μM; Sigma) -sensitive as routinely determined at the end of experiments. Tissue strip weights were measured and recorded at the end of each experiment.

2.4. Drugs

All compounds were purchased from Sigma (St. Louis, MO), unless specified, and included: a) atropine, a muscarinic receptor antagonist, b) guanethidine, an inhibitor of norepinephrine release, c) Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS enzyme that produces NO, d) Phenylephrine (PE), an alpha1 receptor agonist, and e) Sodium nitroprusside (SNP), an NO donor. Stock solutions for all compounds were made in water and added directly to the bath for final working concentrations.

2.5. Experimental protocol (Fig. 1)

An initial baseline tension of 0.5 g was applied to each urethral strip (1 g for bladder) and tissue was washed every 15–20 min for ~1–2 h until stable baseline was achieved. Tissue was tested for viability using high KCl (80 mM) stimulation. Following the application of KCl, a series of 2–3 washes over ~30 min were performed until responses returned to baseline tension values. Each experiment was divided into six steps (Fig. 1). In step 1, baseline EFS responses, which consisted of mixed contractions/relaxations were recorded. EFS frequency (1, 2, 5, 10, 20 Hz)- and voltage (10–80 V at 5 Hz) -response curves were constructed and EFS intensity was adjusted at this time to evoke a response of approximately half maximal amplitude at 5 Hz frequency. In step 2, while maintaining EFS at 5 Hz, atropine (1 μM) and guanethidine (10 μM) were added to block cholinergic and adrenergic-induced responses in order to isolate EFS-induced relaxations. These relaxations are also called nonadrenergic noncholinergic (NANC) relaxations. In step 3, PE (10 μM) was added to pre-contract the strips while maintaining 5 Hz EFS stimulation. The PE-induced contraction was relatively stable for the remainder of the experiment, which typically lasted ~2.5 h. In step 4, three concentrations of L-NAME (1, 10, 100 μM) were cumulatively applied, every 20 min, to test the nitrergic nature of EFS-induced relaxations. In step 5, three concentrations of the NO donor SNP (0.01, 0.1, 1 μM) were added to test the ability of smooth muscle to relax in response to NO. In step 6, the final resting tension recorded in response to 1 μM SNP was compared to the initial baseline tension recorded in step 1.

In our preparation, the urethra strips contain the smooth muscle and striated muscle. The striated muscle was more prominent in the mid urethra. While we cannot entirely exclude the contribution of the striated muscle to urethral relaxations and contractions induced by pharmacological and electrical field stimulation used in these experiments, evidence from literature outlined below, suggests that the responses observed are likely from the smooth muscle. First, there is little evidence supporting direct effects of adrenergic receptor agonists on the striated

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