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RESEARCH ARTICLE

Enhanced production of nitric oxide may contribute to the induction of detrusor underactivity in sucrose fed rats

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Abbreviations used: DU, detrusor underactivity; NO, nitric oxide; SF-2W, two weeks sucrose feeding; SF-4W, four weeks sucrose feeding; EFS, electrical field stimulation; CCh, carbachol; abMe-ATP, alpha, beta-Methylene-ATP; LUTS, lower urinary tract symptoms; UAB, underactive bladder; mN, mili-Newton; NOS, nitric oxide synthase; NOX, both nitrites and nitrates levels indicative of NO quantities

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ABSTRACT

OBJECTIVE: In a previous study we showed that sucrose feeding creates cystometric properties resembling detrusor underactivity (DU) associated with enhanced production of nitric oxide (NO) in rats. Our present objective was to evaluate the possible role of P2X-purinergic and cholinergic receptors as molecular mechanisms underlying DU-like conditions associated with enhanced production of NO and altered contractile properties in rat bladder strips.

MATERIALS AND METHODS: Female Sprague-Dawley rats had ad-libitum access to 5% sucrose in water for two to four weeks. Isometric detrusor contractions were characterized in response to electrical field stimulation, the cholinergic agonist carbachol, the P2X agonist alpha, beta-methylene-ATP or KCl-induced depolarization. ATP and NO release rates were determined from bladder strips after stimulation of cholinergic or P2X-type purinergic receptors.

RESULTS: Electrical field stimulations generated stronger contractile responses in the strips from sucrose fed animals, while KCl induced similar contractions. The release of ATP after cholinergic or purinergic stimulation was similar in control or sucrose fed bladder strips. Nonetheless, NO production was significantly higher when pharmacologically triggered with either cholinergic or purinergic stimulation in the bladder strips from rats drinking sucrose for four weeks. The contractile responses to carbachol or abMe-ATP were also significantly reduced in this group.

CONCLUSIONS: Detrusor contractions in these underactive-like conditions are gradual, and may be generated by excessive NO production that is mediated by individual activation of cholinergic or purinergic receptors, with higher effects by the latest. Our results suggest that nitric oxide production and not impaired contractile mechanisms are involved in this rat model of detrusor underactivity.

Keywords: adenosine-triphosphate, cholinergic receptor, detrusor underactivity, nitric oxide, purinergic receptor

INTRODUCTION

The quality of life for patients suffering lower urinary tract symptoms (LUTS) is decreased by either overactivity or bladder underactivity (UAB), where the latter is a less understood disorder [1], besides detrusor underactivity (DU) remaining a well-defined problem of cystometric voiding performance [2]. Independently of the bladder dysfunction, the micturition reflex involves the coordinated activity of afferent and efferent neural pathways to induce relaxation of the urethral sphincter and simultaneous contraction of the detrusor to expel urine from the bladder [3]. In some forms of LUTS the sensory signals may work as expected to alert the patient as the bladder fills, however, the detrusor muscle may provide a reduced contractile strength leading to failure to void or empty incompletely [4]. Frequently, DU and UAB conditions are observed during neurologic disorders and myogenic failure associated with aging and diabetes [4]. Nevertheless, many of the bladder cellular and regulatory pathways affected during UAB need more intense evaluation for designing better clinical management and lessen bothersome

daily self-catheterization [1,4].

Different transmitters released from urothelial cells and bladder nerves activate either relaxation or contraction pathways for proper urination [5]. Alterations in the bladder levels of some of these transmitters have been considered as useful biomarkers for assessing LUTS [6]. For instance, detrusor overactivity, bladder outlet obstruction or painful bladder syndrome usually show alterations in the normal levels for ATP, nerve growth factor or NO [7-10]. Although DU can be observed in disorders where either the efferent or the afferent branches of the micturition reflex have been impaired [11], other potential changes in sensory transmitters are poorly understood [2].

Impaired production of NO and increased release levels of ATP may be associated with detrusor overactivity in a spinal cord injury model [10], while on the opposite side, increased NO production may be related to the development of DU in rats when supplementing the drinking water with sucrose [12]. To better understand whether purinergic and cholinergic receptors participate in the pathophysiology of DU by modulating the production of NO as a transmitter inducing detrusor relaxation, we characterized detrusor contractions and transmitter release (ATP, NO) in longitudinal bladder strips isolated from rats fed with 5% sucrose that drive the development of a DU-like condition.

MATERIALS AND METHODS

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Houston Methodist Research Institute, and completed in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

DU-like model

As performed in previous studies [12,13], female Sprague-Dawley rats (250-300 g body weight) received 5% sucrose *ad libitum* in water for a period of either two (SF-2W) or four weeks (SF-4W); control rats received regular tap water. All animals were fed with a standard chow diet while maintained under a 12 h light: dark cycle. Blood glucose levels, obtained from a tail vein, were determined at the end of the evaluation using an OneTouch glucometer (LifeScan, Milpitas, CA). Bladder weights were calculated from the control and the SF-4W, while body weights were recorded for all three groups.

Detrusor contractile responses

Isometric bladder-strip contractions were performed in control, SF-2W, and SF-4W animals (N = 6 per group). Rats were euthanized with an intraperitoneal overdose of urethane (2 g/kg) and the whole urinary bladder placed in oxygenated Krebs solution containing (in mM): 113 NaCl; 4.7 KCl; 1.25 $CaCl_2$; 1.2 $MgSO_4$; 25 $NaHCO_3$; 1.2 KH_2PO_4 and 11.5 D-Glucose oxygenated with a 95% O_2/5% CO_2 carbogen gas mix to maintain pH at 7.4. The urinary bladder was cut longitudinally into two strips; each half was used for evaluating either cholinergic or purinergic contractile responses. Each strip was mounted in an organ bath filled with oxygenated Krebs at 37°C. Platinum electrodes were used for electrical field stimulation (EFS). The isometric condition was achieved by applying a pre-tension of 10 mN to each strip before carrying out any contractile procedure. Detrusor contractions were measured with force transducers connected to a bridge amplifier (both from WPI, Sarasota, FL). WinDaq software (DataQ, Akron, OH) was used for data acquisition at a rate of 80 Hz. The contractile response to EFS (100 V/32 Hz/0.25 ms/10 s every 120 s) was determined for 12–15 minutes (i.e. at least 6 contractions). The Krebs solution in the organ baths containing the strips was washed-out and the contractile responses to cholinergic receptor activation were evaluated by applying carbachol (CCh) at a final 50 µM concentration to one half of the bladder; only the steady-state CCh-induced contraction level was considered for the present evaluation. Similarly, a transient P2X-mediated contractile response of the detrusor muscle towards 50 µM α,βMe-ATP was elicited on the other bladder half, and the peak force response before desensitization recorded for later analysis. After a second wash-out with fresh oxygenated Krebs, the contractile response to high potassium depolarization was determined by changing the KCl concentration in the organ baths to 140 mM without adjusting molality as this was the last step of the stimulation. In all stimulation cases, contractile values were calculated as the generated force in mili-Newton (mN), but normalized by their corresponding response to KCl depolarization for normalization and further analysis.

Bladder release of ATP and NO

Transmitter release was chemically stimulated from additional groups of control, SF-2W, or SF-4W bladders (also N = 6 rats per group and different from those used in contractile studies). Urothelium-intact detrusor strips were tied at each end with 5-0 silk sutures and secured, without excessive but similar stretching, inside a 1 ml organ bath maintained at 37°C. The detrusor strip placed inside the organ bath was perfused with oxygenated Krebs at a continuous rate of 1 ml/min. Briefly, to chemically isolate carbachol and P2X-induced responses, we performed a continuous stabilizing perfusion for 30 minutes. After this equilibration time, baseline perfusate collection of 1 ml per minute started for three minutes before changing the perfusion solution to one containing 50 µM CCh; this was perfused for three additional minutes (i.e. cholinergic stimulation) before returning to regular Krebs. Perfusate collection was continued for 5 additional minutes to save a total of 8 samples for determination of transmitter concentrations. The same stimulation and collection procedure was performed using 50 µM ab-MeATP for stimulating P2X purinergic receptor activation. ATP contents in the 50 µM ab-MeATP solution were close to zero (i.e. versus regular Krebs) and significantly lower than those values following the cholinergic or purinergic stimulation ($P < 0.001$). In all cases, collected Krebs samples were stored at -80oC until evaluation of transmitter release approximately one week later, when samples from two or more conditions were available.

ATP and NO determinations

Total ATP concentrations were calculated with a high sensitivity bioluminescence luciferase assay kit (Roche Diagnostic Co., Indianapolis, IN). The total levels of nitrite and nitrate (NOX) were evaluated using vanadium (III) chloride with a Sievers Nitric Oxide Analyzer (GE Analytical Inst., Boulder, CO) and considered as a direct indication of total NO production. We found high variations when determining the raw NO or ATP concentrations among bladder strips, thus decided to normalize both NO as well as ATP concentration values by the corresponding collection time and the individual strip weight as a way to characterize a most reliable response.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Statistical analysis

An unpaired student t-test was performed between control and SF-4W groups to analyze bladder weights. For body weight, blood glucose levels, detrusor contraction, and transmitter release studies, ordinary one-way ANOVA tests were performed between control, SF-2W, and SF-4W groups followed by a Tukey's multiple comparison test. Additional one-way ANOVAs were carried out for NO production after cholinergic or P2X-purinergic activation on each experimental group. In all cases, statistical significance was considered when $P < 0.05$. Figures as well as statistical group analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA). Data are presented as mean-value ± standard error of the mean (M ± SEM), with statistical significance indicated in the corresponding figure legend.

RESULTS

General characteristics after sucrose feeding

Continuous sucrose consumption did not induce substantial blood glucose increases in control (113.5 ± 4.5 mg/dL), SF-2W (122.0 ± 9.9 mg/dL) or SF-4W (120.0 ± 9.5 mg/dL) animals. In comparison with control rats (256.5 ± 4.7 g), body weight was increased in SF-2W (284.3 ± 8.9 g) and SF-4W (284.5 ± 8.6 g) without reaching statistical significance after ANOVA analysis followed by a Tukey's test (P = 0.0518 for SF-2W vs control, and P = 0.0504 for SF-4W vs control). Nonetheless, bladder weight in the SF-4W group was significantly higher than in control animals (103.6 ± 3 vs 159.7 ± 11 mg; $P < 0.01$ by t-test).

Detrusor contractions in response to electrical field stimulation or high potassium depolarization

High KCl depolarization generated detrusor contractions mediated by the activation of voltage-gated calcium channels that were indistinguishable in control, SF-2W, or SF-4W bladder strips (**Fig. 1A**). On the other hand, neurogenic contractile responses to EFS, which promotes the release of excitatory transmitters from nerve terminals, were increased during DU-like conditions in a time dependent manner (**Fig. 1B**).

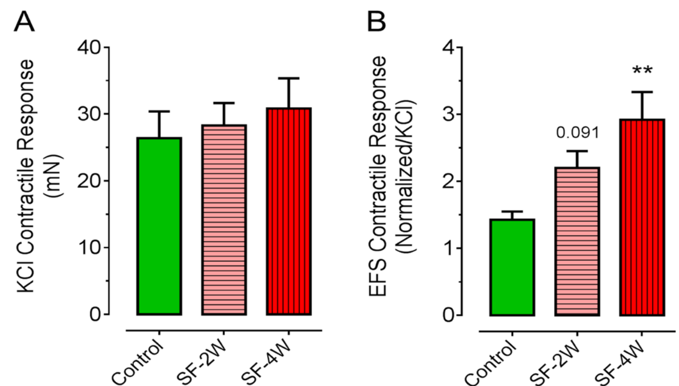

Figure 1. DU-like responses during depolarizing and neurogenic detrusor contractions. A. Contractile response to high potassium depolarization in control, SF-2W and SF-4W rat bladder strips. **B.** Neurogenic contractile responses to electrical field stimulation (EFS; normalized by corresponding KCl contraction) in control, SF-2W and SF-4W rat bladder strips. ANOVA followed by Tukey's test indicate $^{**}P < 0.01$ for control vs SF-4W. N = 6 per group.

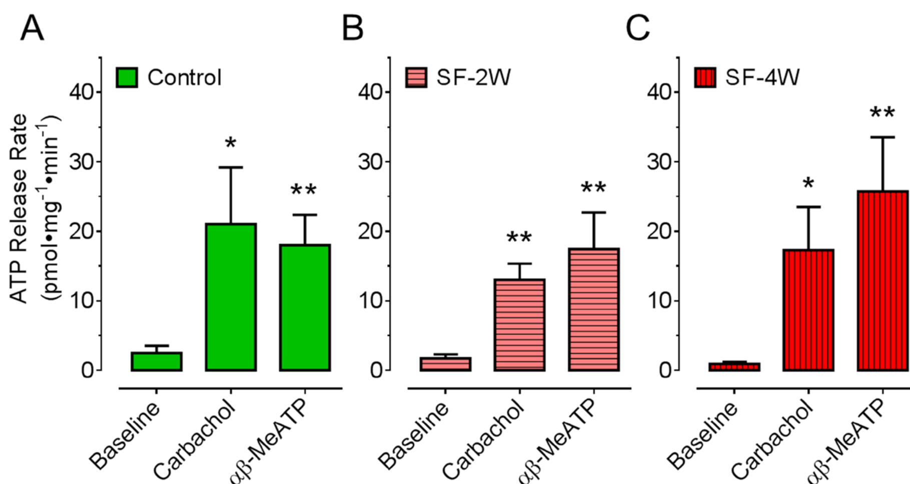

Figure 2. DU-like conditions do not disturb bladder ATP release induced by cholinergic or P2X-purinergic stimulation. A. ATP release rate from control bladder strips in response to cholinergic receptor activation with carbachol or P2X-mediated purinergic receptor activation with abMe-ATP. **B.** ATP release from SF-2W bladder strips after cholinergic or P2X-purinergic activation. **C.** ATP release from SF-4W bladder strips after carbachol or abMe-ATP application. ANOVA followed by Tukey's test indicate $^{*}P < 0.05$ and $^{**}P < 0.01$ for carbachol, or abMe-ATP application vs baseline release. N = 6 per group.

ATP release in response to cholinergic or P2X-purinergic stimulation

The activation of cholinergic receptors with CCh generated a significant release of ATP from control (**Fig. 2A**), SF-2W (**Fig. 2B**), or SF-4W (**Fig. 2C**) bladder strips when compared with their corresponding baseline levels. Similarly, activation of P2X-type purinergic receptors with abMe-ATP generated an important release of ATP in the three groups, with levels comparable to those observed during cholinergic stimulation (**Fig. 2A**-C). Baseline ATP levels seem to be smaller in the SF-4W groups but no statistical significance was detected.

NO production in response to cholinergic or P2X purinergic stimulation

With respect to the baseline levels, activation of either cholinergic or P2X-mediated purinergic pathways generated a significant increase in the production of NO from control (**Fig. 3A**), SF-2W (**Fig. 3B**), and SF-4W bladder strips (**Fig. 3C**). Nonetheless, NO production in the SF-4W bladder strip group was significantly higher in response to carbachol ($P < 0.05$) or abMe-ATP ($P < 0.01$) than in both the control and the SF-2W groups (**Fig. 3C**). In the SF-4W group the baseline NO production seems to be higher but no statistical significance was detected when comparing with the control group.

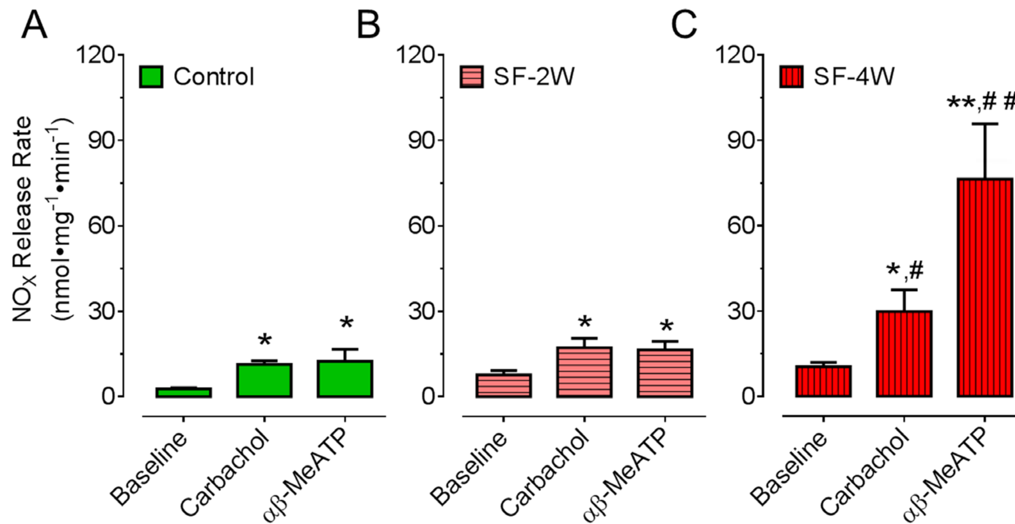

Figure 3. DU-like conditions increase bladder NO production induced by cholinergic or P2X purinergic receptors stimulation. A. NOX production rate from control bladder strips in response to cholinergic receptor activation with carbachol or P2X-mediated purinergic receptor activation with abMe-ATP. **B.** NOX production rate from SF-2W bladder strips after cholinergic or P2X-purinergic activation. **C.** NOX production rate from SF-4W bladder strips after carbachol or abMe-ATP application. ANOVA followed by Tukey's test indicate $^*P < 0.05$ and $^{**}P < 0.01$ for carbachol, or abMe-ATP application vs corresponding baseline release; $\#P < 0.05$ for SF-4W vs either control or SF-2W during carbachol application and $\#\#P < 0.01$ for SF-4W vs either control or SF-2W during abMe-ATP application. N = 6 per group.

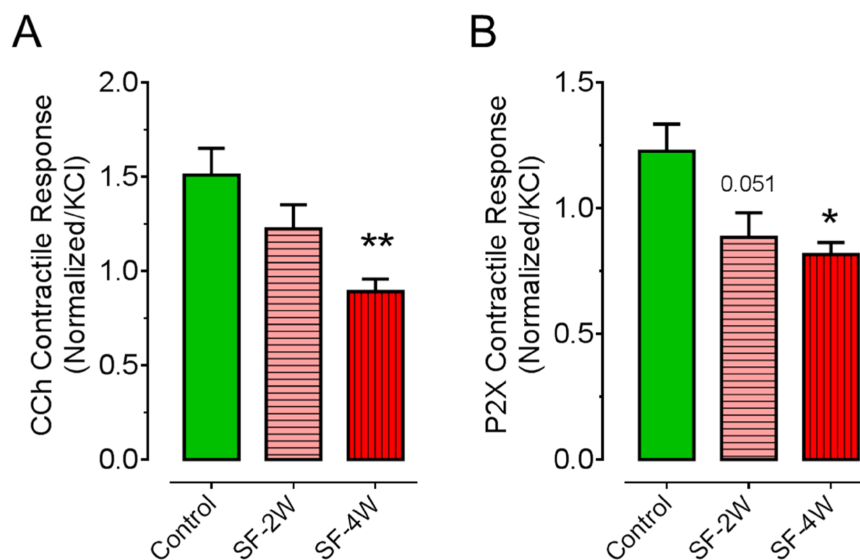

Figure 4. Cholinergic and purinergic detrusor contractions are decreased during DU-like conditions. A. Contractile detrusor response to carbachol application in control, SF-2W and SF-4W rat bladder strips. **B.** Contractile detrusor response to abMe-ATP application in control, SF-2W, and SF-4W rat bladder strips. ANOVA followed by Tukey's test indicate $^*P < 0.05$ for control vs SF-4W in response to carbachol, and $^{**}P < 0.01$ for control vs SF-4W in response to abMe-ATP. N = 6 per group.

Cholinergic and P2X-purinergic detrusor contractions

In comparison with control strips, cholinergic contractions of the detrusor muscle were somewhat reduced in the SF-2W group, but reached statistical significance in the SF-4W (**Fig. 4A**). On the other hand, when compared with control strips, activation of P2X-type purinergic receptors with abMe-ATP generated reduced detrusor contractions in both the SF-2W and the SF-4W groups (**Fig. 4B**).

DISCUSSION

Increased fluid intake and subsequent urine output during sucrose feeding may lead to DU by dysregulation of vascularization due recurrent distensions [14]. Our results suggest that sucrose-feeding in rats may trigger bladder molecular mechanisms that could lead to DU-like conditions characterized by 1) increased NO production in response to cholinergic and P2X-purinerigc stimulation; and 2) decreased cholinergic and P2X-purinergic contractile responses that can be associated with the higher production of NO. In other words, rats with DU-like disorders may have normal strip contraction strengths but increased NO production favoring a more aggravated state for developing an UAB condition. Our results confirm sucrose feeding as an effective way to induce diuresis in rats [15] accompanied with conditions resembling the clinical scenario for impaired detrusor contractions [4,12], and further support a mechanism during development of UAB involving nitrergic pathways.

At the evaluated time points of this study, the effects caused by sucrose ingestion on bladder function may be associated with increased drinking volumes leading to larger bladders, without activating oxidative stress pathways, a situation observed in rats with lumbar canal stenosis or diabetes [16,17]. Accordingly, the detrusor hypertrophy detected during sucrose feeding [14], but not oxidative cell or nerve damage, strongly supports the hypothesis of altered regulation of transmitter release sources leading to underactive bladder dysfunction [12]. In fact, the contractile response to a high potassium depolarization that activates voltage-gated calcium channels [18], suggests that the detrusor contraction systems were not affected by DU-like conditions. This result further supports our previous observation that voiding contractions were smaller in SF-4W animals during open cystometry under urethane anesthesia[12]. Therefore, an increase in the release of inhibitory factors inducing DU, for instance NO, may explain why voiding contractions are reduced concomitantly with an increase in the intercontractile interval.

An obvious contradiction to our previous *in vivo* observations in rats with DU-like conditions [12] is the finding that detrusor contractions induced by EFS were higher in the SF-4W bladder strips. Unfortunately we did not block either the cholinergic (i.e. by using atropine or 4-DAMP) or the purinergic (i.e. by using suramin) contractile components during EFS in order to determine their relative contributions. We speculate that the higher contraction response may be associated, for instance, with the reported bladder hypertrophy during diuresis [12,15], creating a situation where the release of different excitatory transmitters, including acetylcholine and ATP from nerve terminals in the bladder, could be acting on a higher number of detrusor smooth muscle cells expressing cholinergic and P2X-purinergic receptors, thus accounting for the increased EFS contractile response. On the other hand, elevated expression levels for muscarinic receptors have been observed during bladder hypertrophic conditions [19]; consequently, enhanced cholinergic receptor activation during EFS may be anticipated. Similarly, increased release of ATP from nerve sources during EFS [20] could facilitate stronger detrusor contractions in association with the increased muscle mass and action on other purinergic receptors not involved in NO production (see below). In the normal rat bladder, the post-junctional release of ATP produces a transient activation of P2X1 receptors on smooth muscle cells that can generate contractions of a considerable magnitude, which are also increased during pathological bladder conditions [21,22].

The bladder release of ATP after cholinergic or P2X-purinergic receptor activation has been well documented [23,24]. In the current study we did not find any significant difference in the ATP release rate from control or sucrose-fed bladder strips after stimulation with either CCh or abMe-ATP. Conversely , the enhanced NO production mediated by activation of cholinergic receptors in SF-4W strips may be mostly originated in the urothelium [24]; similarly, abMe-ATP can activate, besides P2X1 receptors in smooth muscle cells, other P2X-type receptors. In fact, the activation of P2X receptors by ATP has been associated with triggering the production of NO via calcium influx and activation of the neuronal-Nitric Oxide Synthase (NOS) isoform in inner hair cells [25]. Thus, the application of abMe-ATP to bladder strips may also activate other P2X-type receptors, particularly P2X3 receptors, in the urothelium [26] and bladder nerve terminals [27] to generate a substantial production of NO in a comparable condition as the one suggested from the studies using inner cells [25].

In our previous report using the same sucrose feeding model but stimulating NO production with a hypoosmotic shock, which creates a physical stimulation of the bladder that mimics bladder distension, higher NO levels were also observed [12]. Using this physical method for bladder stimulation the stretch-facilitated release of acetylcholine and ATP could be expected to activate nitrergic pathways for higher NO production, and thus reinforcing our current observations. In the end, the differences observed between cholinergic- and purinergic-mediated productions of NO may be related to the corresponding receptor activation in specialized cells expressing nitric oxide synthases for the production of this inhibitory transmitter.

Our results suggest that cholinergic, but mainly P2X-purinergic receptors, may play a role in the development of DU-like conditions in rats drinking sweetened water. These predictions are supported in DU-like bladder strips during the chemical-induced contractions. We speculate that the cholinergic and purinergic contractile amplitudes could be lower than those induced by EFS because only cholinergic (activated with carbachol) or purinergic (stimulated with abMe-ATP) receptors were primarily activated. The higher production of NO following the separated activation of these receptors may also account for the reduction in the contractile responses independently of bladder hypertrophy. The unchanged ATP release in SF-4W strips during P2X activation might seem contradictory for attenuated P2X1-driven detrusor contractions; however a cryoinjury model that induces impaired detrusor function also found that the contractile strip responses to abMe-ATP were reduced in the DU-like strips from rats [28]. Based on that report and our present observations, it is plausible to suggest that P2X-type purinergic receptors participate, in a paradoxical way, on generating detrusor contractions but at the same time further enhancing the production of NO during development towards a DU-like bladder dysfunction.

CONCLUSIONS

The use of rats fed with sucrose allowed us to verify the presence of dysfunctional detrusor contractions that can be explained by an increased production of NO. This inhibitory transmitter can generate DU-like conditions by amplifying detrusor relaxation independent of either cholinergic or P2X-purinergic stimulation. Although our results provide some insight into the impaired contractile pathways during DU, they also raise questions about what NOS isoforms are overexpressed and/or post-translationally modified along with a possible role of urothelial cells. Ultimately, a better understanding of the pathological processes leading to DU associated with UAB is required to target development of therapeutic treatments.

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