Alterations in neurogenically mediated contractile responses of urinary bladder in rats with diabetes

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Liu, Guiming, and Firouz Daneshgari. Alterations in neurogenically mediated contractile responses of urinary bladder in rats with diabetes. Am J Physiol Renal Physiol 288: F1220–F1226, 2005—Diabetic bladder dysfunction (DBD) is among the most common and bothersome complications of diabetes mellitus. Autonomic neuropathy has been counted as the cause of DBD. In the present study, we compared the alterations in the neurogenically mediated contractile responses of urinary bladder in rats with streptozocin-induced diabetes, 5% sucrose-induced diuresis, and age-matched controls. Male Sprague-Dawley rats were divided into three groups: 9-wk diabetic rats, diuretic rats, and age-matched controls. Micturition and morphometric characteristics were evaluated using metabolic cage and gross examination of the bladder. Bladder detrusor muscle strips were exposed to either periodic electrical field stimulation (EFS) or to EFS in the presence of atropine, α,β-methylene adrenasine 5′-triphosphate, or tetrodotoxin. The proportions of cholinergic, purinergic, and residual nonadrenergic-noncholinergic (NANC) components of contractile response were compared among the three groups of animals. Diabetes caused a significant reduction of body weight compared with diuresis and controls, although the bladders of diabetic and diuretic rats weighed more than the controls. Both diabetes and diuresis caused significant increase in fluid intake, urine output, and bladder size. Diabetes and diuresis caused similarly increased response to EFS and reduced response to cholinergic component compared with controls. However, the purinergic response was significantly smaller in diuretic bladder strips compared with controls but not in diabetic rats. A residual NANC of unknown origin increased significantly but differently in diabetics and diuretics compared with controls. In conclusion, neurogenically mediated bladder contraction is altered in the diabetic rat. Diabetic-related changes do not parallel diuretic-induced changes, indicating that the pathogenesis of DBD needs further exploration.

Bladder contraction is mediated by both neurogenically mediated cholinergic and nonadrenergic-noncholinergic (NANC) pathways (3). Muscarinic receptor stimulation, cholinergic, is responsible for the main part of bladder emptying. ATP, the main NANC excitatory transmitter, induces purinergic contraction. Besides, it also became evident that motor responses that are partially mediated by some other NANC neurotransmitters and/or receptors take place in the bladder (46). Several studies have demonstrated that the cholinergic and purinergic components changed in pathological detrusor muscle obtained from patients with benign prostate obstruction, detrusor instability, interstitial cystitis, and chronic paraplegia (40, 42, 54). Whatever the cause, the possibility of a change in the neurotransmission pattern in abnormally functioning bladders is of great importance as it offers a potential target for drug development aimed at dysfunctioning tissue (4). Whether a similar change occurs in the urinary bladder of diabetic patients, it would have an important effect on pharmacological management of detrusor impairment. Irritable symptoms, including urinary frequency and urgency, in patients with diabetes are bothersome and sometimes resistant to cholinolitics (19). Non-cholinergic components may therefore be involved. It is clearly of some interest to determine whether these components are altered in the neurogenically mediated contractile response of urinary bladder in rats with diabetes.

Additionally, diabetes leads to increased fluid intake (polydypsia) and urine output (polyuria) by the virtue of its induced hyperosmolar status (33). This imposes an extra demand on the bladder to void the excessive excreted urine. Experimentally induced diuresis in both rats and rabbits causes bladder hypertrophy, increased contractility, increased capacity, and increased compliance that is similar to that observed in diabetic rats (12, 48). The similarities between the findings in diabetic and diuretic rats suggest that the bladder hypertrophy in diabetic animals may be the result of a physical adaption to increased urine production and that the changes in the physical properties of the bladder may be a significant factor in the development of vesical dysfunction in diabetes (28). In this study, we will identify the extent to which diuresis alters neurogenically mediated contraction in diabetic animals.

The current study was designed to investigate the relative contribution of these distinct neurotransmission signaling: cholinergic, purinergic, and the residual NANC components, to the neurogenically mediated contraction of the bladder in rats with induced diabetes and diuresis compared with age-matched controls. These components were relatively separated from each other by pharmacological means.

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MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats matched by date of birth (240 to 260 g, 8 wk old, Harlan) and housed in a 12:12-h light-dark facility with food and water provided ad libitum were used in this study. The animals were randomly allocated to three groups of equal numbers \( (n = 12): 9\)-wk diabetic rats, diuretic rats, and age-matched controls. Diabetes was induced in the rats by intraperitoneal injection of streptozocin (STZ; 65 mg/kg dissolved in 0.1 M citrate buffer), and diuresis was induced by addition of 5% sucrose to their drinking water. Blood samples were taken 72 h after administration of STZ to confirm diabetes (blood glucose >300 mg/dl). Blood glucose levels were measured with the ACCU-CHEK advantage blood glucose monitoring system (Roche Diagnostics, Indianapolis, IN). All experimental protocols and procedures were approved by the Cleveland Clinic Foundation Institutional Animal Care and Use Committee (Cleveland, OH).

Micturition. At 9 wk posttreatment, metabolic characteristics were measured for all rats. Rats were placed in individual metabolic cages (Nalgene, Nalge, New York) and the previous food, water, and light-dark conditions were maintained for a minimum of 24 h. Following this familiarization period, a known volume of water or 5% sucrose was placed in the drinking bottles. At the end of 24 h, the volume of liquid remaining in the drinking bottles was measured, and urine was collected. The volume of liquid consumed was calculated, and the voided volume was measured for each treatment group.

Bladder contractility experiments. All rats were weighed and then humanely decapitated (under 4% isoflurane inhalation anesthesia). Blood samples were collected immediately to measure blood glucose. The bladder was removed at the level of the ureters, blotted, weighed, and placed in Krebs buffer. Half of the animals in each group \( (n = 6) \) were used for contractility and the other half \( (n = 6) \) were used for microscopic examination. For contractility studies, two strips of detrusor muscle from each bladder were used. Full-thickness longitudinal strips (10 × 2 mm), weighing ~10 to 15 mg, were prepared from the dorsal part of the bladder body. The strips were attached to glassy tissue holder stands at one end and to force displacement transducers at the other end. The bladder strips were mounted between platinum plate electrodes (2.5-cm long and 2.0-cm apart) in a double-jacketed organ bath (Radnoti, Radnoti Glass Technology, Monrovia, CA) containing 20 ml of Krebs solution aerated with 95% O\(_2\)-5% CO\(_2\) and maintained at 37°C, pH 7.4.

Following a 45-min equilibration period, the resting tension was adjusted every 10 min, three to five times, to reach optimal length (49). Isometric contraction was recorded at a rate of 50 Hz with a computerized data-acquisition program (Biobench, National Instruments, Austin, TX). After reaching a stable baseline, the contractions were measured at 0.5, 1, 2, 4, 8, 16, and 32 Hz. The contractions were measured at 0.5, 1, 2, 4, 8, 16, and 32 Hz.

To evaluate cholinergic contraction, the tissue preparation was bathed in Krebs solution containing 1 \( \mu \)M atropine for 30 min before the EFS stimulation was repeated. The cholinergic response was measured as the difference in the contraction before and after the addition of atropine. The purinergic component of the residual atropine-resistant contraction was evaluated with desensitization of the P2X purinergic receptor using \( \alpha,\beta\)-methylene-ATP (\( \alpha,\beta\)-MeATP; 10 \( \mu \)M per application) applied three to five times at 10-min intervals until the contraction caused by this agent was absent (34, 54, 55). The frequency-response curve of the remaining residual component was examined in the continuous presence of atropine and \( \alpha,\beta\)-MeATP. Once these agent-resistant contractile responses were observed, tetrodotoxin (TTX; 1 \( \mu \)M) was added to confirm the neurogenic mediation of the response. The proportion of atropine-sensitive (cholinergic) and atropine-resistant, \( \alpha,\beta\)-MeATP-sensitive (purinergic) components of the EFS-induced contraction was determined for each muscle strip at each frequency. The residual response after desensitization of the P2X purinergic receptors was measured as the residual NANC. Matched control strips from the same bladders were treated identically to experimental tissues, except for exposure to antagonists, in a time-controlled manner. At the end of the experiment, the length and weight of each muscle strip were measured between the suspension clips.

Bladder fixation and staining. To characterize the morphological changes in the bladder in diabetic and diuretic rats, the bladders from the second half of each group \( (n = 6) \) were equilibrated in 37°C Krebs solution for 15 min, sectioned at the equatorial midline, and fixed in 10% neutral buffered formalin (pH 7.0). After fixation, tissues were dehydrated and paraffin embedded. Serial 5-\( \mu \)m tissue sections were placed on microscope slides, dewaxed, and rehydrated for routine hematoxylin and eosin staining. The slides were scanned and photomicrographs were captured. The images were analyzed with Image Pro 5.0 image analysis software (Media Cybernetics, Silver Spring, MD). The inner lumen cross-sectional area and wall area (the difference between outer and inter circumference) were measured by tracing the internal and external edges of the bladder.

Drugs and chemicals. The following pharmacological agents were used: STZ, sucrose, atropine (1 \( \mu \)M), \( \alpha,\beta\)-MeATP (10 \( \mu \)M), and TTX (1 \( \mu \)M). Other chemicals and materials were of analytic grade. The composition of the Krebs buffer was as follows (in mM): 133 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.35 NaH\(_2\)PO\(_4\), 0.6 MgSO\(_4\), 16.3 NaHCO\(_3\), and 7.8 dextrose. All agents were purchased from Sigma (St. Louis, MO).

Statistical analysis. Contraction responses to EFS are expressed as tension \( (g) \) per cross-sectional area \( (mm^2) \) ± SE. The cross-sectional area of the bladder strip was calculated as mass/length \( \times \) density, where 1.05 is the assumed density of the bladder strip (32). The cholinergic component was calculated per frequency as the portion of the total contraction inhibited by atropine, divided by the proportion of the total contraction in each muscle strip; the purinergic response was calculated per frequency as the proportion of atropine-resistant, \( \alpha,\beta\)-MeATP-sensitive portion of the total contraction, divided by the proportion of the total contraction in each muscle strip; the residual NANC component was calculated per frequency as the portion of the total contraction inhibited by atropine and desensitized by \( \alpha,\beta\)-MeATP, divided by the proportion of the total contraction in each muscle strip. Statistical analysis was performed with SPSS 11.5 analysis software (SPSS, Chicago, IL). General characteristics between groups were compared using one-way ANOVA, followed by a Bonferroni post hoc test. Comparisons of contraction responses and components were performed by repeated-measures ANOVA followed by a Bonferroni post hoc test. Probability values of <0.05 were considered to be statistically significant.

RESULTS

General characteristics. General physical characteristics of the animals were determined 9 wk after induction of diabetes and diuresis (Table 1). The initial mean body weight was similar for all three groups, but at 9 wk the diuretic and control groups weighed ~51 and 50% higher than the diabetic group, respectively. There was no significant difference between the body weights of the diuretic and control animals \( (P = 0.848) \). The ratio of bladder weight/body weight was about 0.2, 0.5, and 1.0 mg/g in age-matched control, diuretic, and diabetic rats, respectively. The mean blood glucose levels of the diabetic rats \( (565.2 \pm 6.9 \text{ mg/dl}) \) were about four to five times higher than those of age-matched control \( (109.5 \pm 7.0 \text{ mg/dl}) \) and diuretic rats \( (103.3 \pm 4.9) \). Both diabetic and diuretic rats showed increased water consumption and urine output. The 24-h urine outputs of diuretic and diabetic rats were signifi-
cantly greater than those of control animals (P < 0.01), as was the volume of fluid intake (P < 0.01).

**Bladder histology.** Histological examination using light microscopy showed hypertrophic changes in the bladder of the diabetic and diuretic animals (Fig. 1). The mean cross-sectional area of the bladder lumen increased in diabetic and diuretic animals compared with controls (5.3 ± 0.7, 5.1 ± 0.6, and 3.5 ± 0.3 mm², respectively; P < 0.05). Also, the bladder wall area increased in diabetic and diuretic animals compared with controls (17.0 ± 1.3, 18.2 ± 1.5, and 11.3 ± 0.8 mm², respectively; P < 0.05).

**Response to EFS.** Frequency-dependent increases in EFS-evoked contractions occurred in all three groups (Fig. 2). The contractile responses to EFS stimulation were increased in the detrusor muscle strips from the diabetic and diuretic groups compared with the control group (P < 0.01). There was no significant difference of the EFS-induced contractions between diabetic and diuretic groups (P = 0.847).

**Cholinergic and NANC components of neurogenically induced contraction.** Figure 3 shows a representative tracing of two detrusor strips from one control animal (Fig. 3, A and B), one strip from a diabetic (Fig. 3C) and one from a diuretic animal (Fig. 3D) undergoing repeated EFS in the absence (Fig. 3A) or presence of pharmacological agents (Fig. 3, B, C, D). Addition of 1 μM atropine left the atropine-resistant component of the contractility response to field stimulation. In the presence of atropine and desensitization with α,β-MeATP, a residual component of contractility response to EFS of unknown origin was observed. This residual component was abolished by TTX (1 μM) and was therefore of neurogenic origin. The frequency-response relationship for the time-matched strip preparation did not differ significantly during repeated EFS (P = 0.920; Fig. 3A), which meant the contractility of the detrusor strip did not change during the experimental period.

The cholinergic component increased with increasing frequency of EFS and was significantly smaller in diabetic and diuretic than in control muscle strips (P < 0.05; Fig. 4A). At 0.5 Hz, the cholinergic component accounted for 24% of the contraction in the control group, 7% in the diabetic group, and 5% in the diuretic group, while at 32 Hz, it was 42, 34, and 36%, respectively. The purinergic component decreased with increasing frequency and was significantly smaller in muscle strips from diabetic animals compared with control (P < 0.05) and diabetic animals (P < 0.05; Fig. 4B). However, there were no differences in the purinergic component between diabetic and control animals (P = 0.923). At 0.5 Hz, the purinergic component accounted for 76% of the contraction in the control group, 84% in the diabetic group, and 45% in the diuretic group, whereas at 32 Hz, it was 55, 49, and 28%, respectively. The residual NANC component of unknown origin accounted for <5% of the contraction in the control rat bladder. However, the residual NANC component increased significantly in diabetic and diuretic muscle strips with controls (P < 0.05), and diuretic animals increased more than the diabetic group (P < 0.05). At 0.5 Hz, the residual NANC component was 0% in the control group, 8% in the diabetic group, and 50% in the diuretic group, whereas at 32 Hz, it was 3, 17, and 36%, respectively (Fig. 4C).

**DISCUSSION**

Diabetes causes marked and yet poorly defined changes in the morphometry and function of the bladder. The most prominent changes include enlargement of the bladder, and increasing fading of its emptying ability (25). In exploring of pathophysiological causes of these changes, some have suggested a prominent mechanistic role for the increased urine output associated with diabetes. Therefore, use of a diuretic group is crucial in distinguishing alterations produce by diabetes-induced effects on contractile function from those induced by the putative effect of increased urine output. The bladders of diuretic animals were compared with bladders from diabetic and control animals in this study. The weights of the bladders

![Fig. 1. Representative photomicrograph of hematoxylin- and eosin-stained equatorial section of urinary bladders from age-matched control (A), 9-wk streptozocin (STZ)-induced diabetic (B), and 5% sucrose-fed diuretic rats (C). Scale bar = 1 mm.](http://ajprenal.physiology.org/)

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from the diuretic animals were ~2.5 times of those from control animals. However, diabetic and diuretic rats present similar increases in their bladder weights. This suggests that diuresis alone may alter the physical properties of the organ, a finding that has been described by other investigators (33). In addition, histological examination of sections of bladder tissue shows enlargement of the bladder wall and lumen in both diabetic and diuretic animals. These results indicate that the observed hypertrophy in diabetes is likely to be induced by diuretic effects of the disease. It is not surprising to conclude that such bladder remodeling could inevitably lead to distortion of the normal pattern of innervation, and other local neurogenic and myogenic changes, which may be the causes of DBD (6).

The EFS-induced response in the bladder strips from diabetic and diuretic rats was greater than in age-matched control animals. This is consistent with a number of other reports of enhanced contractile responses in rat bladder after 8 to 12 wk of diabetes (31, 48, 53). However, some other reports concern-
neurogenically induced contraction in diabetic and diuretic animals, including the sensitivities or affinities of bladder smooth muscles to the cholinergic and purinergic neurotransmitters, and the amounts of neurotransmitters released from intrinsic nerves (16, 20).

It is well known that field stimulation of isolated detrusor specimens activates the embedded motor nerve endings and causes a frequency-dependent contraction. There are more than two distinct components to this contraction (52, 55). The major component is inhibited by atropine and is thus mediated by acetylcholine (Ach) via a muscarinic (M) receptor (cholinergic). The second component is inhibited by α,β-MeATP and is mediated by ATP (purinergic). α,β-MeATP, an ATP analog, is resistant to ATPase and selectively acts on the P2X receptor. Thus P2X receptors can be desensitized through repeated application of α,β-MeATP (15). Additionally, there is always a small residual NANC component of detrusor contraction that is not mediated by either M or P2X receptors (22, 56).

In the present study, we separated EFS-induced contraction in rat detrusor smooth muscle strips into cholinergic, purinergic, and residual NANC components by using pharmacological means. In age-matched control rat detrusor, we found that the cholinergic and purinergic components were ~40 and 60% (field stimulation at 8 Hz), which is consistent with previous findings (54). The response to neuromuscular transmission is different with each stimulation frequency. The cholinergic component of nerve-induced contraction increased with increasing frequency, but the purinergic component decreased with increasing frequency. In the presence of atropine and α,β-MeATP, there remained a small residual response (~3%) to EFS, especially at 8, 16, and 32 Hz. This was abolished by TTX and was therefore of neurogenic origin. Similar findings have been described in other reports (22, 56).

The cholinergic component was significantly smaller in diabetic and diuretic than in control muscle strips, whereas the purinergic component was significantly smaller in diuretic, but not in diabetic, compared with control. Additionally, results from our study clearly demonstrate that responses to EFS were inhibited >95% by atropine and α,β-MeATP in detrusor from control animals, in agreement with previous studies (46). However, specimens from diabetic and diuretic animals showed greater residual NANC contractile responses than those from controls in the presence of atropine and α,β-MeATP, particularly in diuretic rats. These responses were completely abolished by TTX, demonstrating that the residual response was neurogenically mediated.

Diuresis alone can induce decreased cholinergic and increased residual NANC components; however, the response from diabetic rats was not consistent with that from diuretic rats in regard to purinergic neurotransmission, suggesting that diuresis is not the only mechanism responsible for the observed changes. Diabetes-induced metabolic alterations appear to be associated with some of the alterations of the neurotransmission pathway of the bladder. Hyperglycemia-induced responses, e.g., autonomic neuropathy, may be partially responsible for the observed alterations. In addition, a decrease in the cholinergic component has also been reported in obstruction-induced hypertrophy of the bladder (8). Therefore, we suggest that diuresis-induced hypertrophy of the bladder may be a major cause of the decrease in cholinergic neurotransmission and the increase in the residual NANA component.
The reason for the pronounced difference in neurotransmission mode among the control, diabetic and diuretic bladders is not clear. In hypertrophied bladders of rat with diabetes, there may be local changes within the bladder wall, for example, in innervation pattern and transmitter contents, in receptor populations, and in the smooth muscle cells. A possible explanation could be that the increase in the residual NANC component is a result of a hypothetical compensatory mechanism for the relative decrease in the cholinergic component of the motor transmission in diabetic and diuretic detrusor.

The nature of the residual NANC component of nerve-induced contraction remains to be identified (17). One possible mechanism is that ATP may act on another type of P2 receptor, but not on the P2X receptor (15). Although multiple P2X receptors have been identified in mice and human bladders, it is generally believed that P2X1 receptors are the predominant subtype involved in the neurally evoked purinergic excitatory effects (39, 50). However, several studies demonstrated that apart from P2X, another type of P2 receptor mediates contraction in rat urinary bladder (15, 41, 45). This type of unknown purinoceptor responds to adenosine 5'-O-(2-thiodiphosphate) (ADPβS) but not to α,β-MeATP (15). Therefore, it cannot be desensitized by repeated addition of α,β-MeATP.

A second possible mechanism involves the existence of other neurotransmitters, such as 5-hydroxytryptamine (5-HT), which has been shown to contract bladder or isolated bladder smooth muscle strips (9, 20). The 5-HT2 receptor is mainly responsible for serotonin-induced contractions of the rat detrusor smooth muscle, whereas the 5-HT1 receptor is partially responsible (26). In addition, a number of neuropeptides that are synthesized, stored, and released in nerves in the detrusor muscle have been identified, but their functional roles have not been fully established (2, 10, 17, 43). Immunohistochemical methods have also demonstrated that the urinary bladder smooth muscle is supplied by nerves containing neuropeptide Y, tyrosine hydroxylase, vasoactive intestinal polypeptide (VIP), galanin, substance P (SP), atrial natriuretic peptide, bradykinin, endothelin (ET)-1, and calcitonin gene-related peptide (18, 23). These molecules may play roles as neurotransmitters and/or neuromodulators at the neuromuscular junctions. It has also been demonstrated that some of these neuropeptides (e.g., VIP, ET, tachykinins, and angiotensins) were involved in detrusor contraction (3, 35). In human ileum in vitro, atropine-resistant contractions to EFS also occur and are inhibited by neurokinin (NK)-2 receptor antagonists, indicating release of a tachykinin (36). A tachykinin-mediated, capsaicin-sensitive component is present in the field stimulation-evoked NANC contraction of the rat bladder detrusor muscle in vitro (37). Taken together, these studies suggest that there are some other components of neuromuscular transmission in nerve-induced contraction apart from the Ach-M and ATP-P2X pathways.

A number of other neurotransmitters and/or receptors may contribute to the increased residual NANC component in nerve-induced detrusor contraction in diabetic and diuretic animals. Previous studies have shown significantly increased contractility of the hyperglycemic rabbit and rat bladder in response to 5-HT (20, 27). These findings suggest that bladder hyperreactivity resulting from hyperglycemia may possibly be attributed to increased density of 5-HT2A receptors on detrusor smooth muscle. In a recent clinical study, Takimoto et al. (47) reported the efficacy of a 5-HT2A receptor antagonist on irritable bladder symptoms resistant to cholinolytics in patients with diabetes. The authors suggested that hyperreflexia of the detrusor in patients with diabetes would be associated with hyperreactivity to 5-HT via the 5-HT2 receptor. Several other endogenous substances, including prostaglandins, have been proposed to be the noncholinergic neurotransmitter responsible for the atropine-insensitive portion of EFS. Kudlacez et al. (28) demonstrated that both diuretic and diabetic bladders showed a twofold increase in maximum pressure response to PGF-2α compared with control, while Steers et al. (44) showed that the amounts of VIP in the bladder were increased in diabetic rats. It has also been demonstrated that the contractile responses to exogenous SP increase significantly in 8- to 11-wk diabetic rats, possibly resulting from denervation supersensitivity (11, 24).

In conclusion, diabetes leads to the alterations of neurogenically mediated contractile pathways in the rat bladder. The cholinergic component of this pathway is diminished, and the residual NANC components are enhanced. Diabetes-associated diuresis accounts for a portion of these changes. Therefore, further investigation of the direct effects of hyperglycemia on the neurogenically mediated contraction of the bladder is warranted.

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REFERENCES