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Alterations of M_2-muscarinic receptor protein and mRNA expression in the urothelium and muscle layer of the streptozotocin-induced diabetic rat urinary bladder

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Abstract

Diabetes associated alterations of M_2-muscarinic receptors (M_2-mAChR) in the urothelium and muscle layer of the urinary bladder were studied using streptozotocin (STZ)-induced diabetic rats. Male Wistar rats were divided into two groups; group I: normal control rats; group II: STZ-induced diabetic rats, 2 weeks after induction. The bladder was divided into urothelium and muscle layer by microdissection. Tissue M_2-mAChR protein levels were measured by Western blotting. Expression of the mRNA that encoded M_2-mAChR was estimated using the method of reverse transcription combined with polymerase chain reaction (RT-PCR). M_2-mAChR protein and mRNA expressions were found in both the urothelium and muscle layer of the rat urinary bladder. In control rats, the M_2-mAChR protein expression ratio in the urothelium and muscle layer was 1:1.66; that for mRNA was 1:0.97. Two weeks after induction of diabetes, the M_2-mAChR mRNA expression in the urothelium and muscle layer were significantly increased by 44.4% ($P<0.01$, $n=8$) and 28.6% ($P<0.01$, $n=8$), respectively. Correspondently, the bladder M_2-mAChR protein levels were significantly increased by 33.3% ($P<0.001$, $n=8$) in the urothelium and 25.3% ($P<0.01$, $n=8$) in the muscle layer of the diabetic rats. In conclusion, M_2-mAChR mRNA and protein are expressed in both the urothelium and muscle layer of the rat urinary bladder. STZ-induced diabetes increases mRNA and protein expression of the M_2-mAChR in the urothelium as well as the muscle layer.

Keywords: Diabetes; Bladder; Urothelium; Muscarinic receptors

The old concept of the urothelium being only a passive barrier between urine and bladder wall is not valid. Research has shown that the urothelium plays an active role in the regulation of bladder activity including: (1) barrier role against chemicals, microorganisms, etc; (2) antigen presentation in host defense mechanisms [2]; and (3) sensory transduction mechanisms [5]. The urothelium exhibits various "neuron-like" properties that contribute to its sensory function. These include: (1) expression of neurotransmitter receptors; (2) release of neurotransmitters by a process resembling exocytosis; (3) expression of stretch-activated channels; (4) release and response to neurotrophic factors; (5) innervation by afferent nerves [1]. It has been proposed that stretching of the urothelium can release transmitters that act on afferent nerves to modulate sensory input from the bladder to the spinal cord. Adenosine triphosphate (ATP), P_{2X3} purinergic receptors on the afferent terminals, and nitric oxide (NO) have been implicated in this urothelial–neural interaction [14].

Lower urinary tract dysfunction (LUTD) is one of the most common complications of diabetes mellitus (DM). In 2-week streptozotocin (STZ)-induced diabetic rats, it has been demonstrated an increased genetic expression of the M_2-muscarinic receptors (M_2-mAChR) in the urinary bladder [20]. Muscarinic cholinergic mechanisms are probably involved in urothelial sensory functions. Evidence indicates that acetylcholine is synthesized and released by urothelial cells and that urothelial cells express muscarinic receptors [4]. In fact, a greater density of muscarinic receptors was found in the urothelium than in the detrusor muscle in the pig bladder [8]. However, the role of urothelium in the pathogenesis of DM-LUTD has seldom been reported. Thus the present study aimed to investigate the DM

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associated alteration of M_2-mAChR expression in the urothelium, as well as that in the muscle layer of the rat urinary bladder.

Male Wistar rats, aged 8–10 weeks weighing 220–250 g, were divided into two groups; group I: normal control rats; group II: STZ-induced diabetic rats, 2 weeks after induction. The rats were kept in a temperature-controlled room (25 ± 1 °C) with 12:12 h light-dark cycle. Food (Purina Rat Chow) and water were available ad libitum. The body weights of the rats were measured daily. The experimental protocol had been approved by the Institutional Animal Care and Use Committee and the animals were handled according to the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised 1996.

An intravenous injection of STZ (Sigma Chemical Co., St. Louis, USA) at 60 mg/kg dissolved in 1% citrate buffer was administered into the femoral vein of 3-day fasting rats under intra-peritoneal pentobarbital anesthesia (30 mg/kg). Control rats received a similar injection of vehicle. Seventy-two hours after induction, serum samples were taken from the tail vein of 8-h fasting rats. The concentration of serum glucose was determined by a glucose analyzer (Quik-Lab, Chemistry Analyzer) using the glucose-oxidase method. The assays were run in duplicate. Rats with serum glucose concentrations of 300 mg/dl or greater were considered as diabetic.

The rats were anesthetized with intra-peritoneal administration of pentobarbital. The bladder was removed via a midline abdominal incision and immediately placed in oxygenated Tyrode's solution (NaCl 125 mM, KCl 2.7 mM, $CaCl_2$ 1.8 mM, NaH_2PO_4 0.4 mM, $MgCl_2{\cdot}7H_2O$ 0.5 mM, $NaHCO_3$ 24 mM and dextrose 5.6 mM). The wet weight of the bladder was measured. The bladder to body weight ratio was calculated as [(bladder weight)/(body weight)] × 100%.The portion of the bladder above the ureteral orifices was harvested as the bladder body. Microdissection technique was used to split the bladder body wall into urothelium and muscle layer. The integrity of the separated urothelial layer was confirmed histologically. For histology study, tissues were placed in 10% formalin for a minimum of 2 days. Standard procedures of paraffin embedding, sectioning (4 μm) and staining with hematoxylin and eosin were carried out.

Tissue M_2-mAChR protein levels were measured by Western blotting using monoclonal antibody. Bladder tissue was lysed in buffer containing 1% Triton X-100. Discontinuous slab gels (1.0 mm thick) containing 0.1% SDS were prepared with acrylamide at concentrations of 12% in the separation gel and 5% in the stacking gel [13]. Protein samples were fractionated by gel electrophoresis run at 40 and 100 V under 4 °C during the stacking and separation steps, respectively. The separated proteins were blotted onto nitrocellulose. After treatment with M_2-mAChR specific antibody (purchased from Affinity Bioreagents Inc., Colorado, USA), immunostaining was preformed for peroxidase activity by incubation in Tris–buffer (10 mM) using enhanced chemiluminescence (ECL) development system (Amersham International, England). Identification of the responses was observed at 60 kDa. The observed Western immunoblots were then quantified by using laser densitometer.

Total RNA was extracted from the bladder tissues using the Ultraspec™-II RNA extraction system. The extracted RNA was dissolved in diethylpyrocarbonate-treated water. The concentration of RNA was estimated by measuring the absorbance at 260 nm. The 260/280 ratio was used for checking purity.

The oligonucleotide primers for amplifying M_2-mAChR mRNA (GenBank accession No. J03025, MWG Biotech, Ebersbery, Germany) were: 5′-AGCCCGCAAAATCGTGAA-3′ (forward primer, position 1534); and 5′-GACATTGTATGGCGCCCAC-3′ (reverse primer, position 1666, product 132 bp) [7]. Total RNA (2 μg) was reverse transcribed in the presence of an anchored oilgo p(dT_{15})-primer (Gibco BRL, Paisley, UK) by the use of AMV reverse transcriptase (Gibco BRL). Single stranded cDNA was amplified by PCR in a 100-μl reaction mixture (pH 8.3) containing 50 mM KCl, 10 mM Tris–HCl, 2 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.25 μM sense and antisense primers and 2.5 units of Tag DNA polymerase (Gibco BRL). The PCR was performed with a Perkin-Elmer GemeAmp PCR system 2400 with the following cycle parameters: one denaturation cycle for 3 min at 95 °C; 35 cycles of 1 min at 94 °C (denaturation), 1 min annealing at 50 °C and 1-min extension at 72 °C. A final extension cycle was run for each reaction for 5 min at 72 °C. Amplification of β-actin mRNA served as an internal standard. The primers used for β-actin were 5′-ATGGTGGGAATGGGTCAGAAG-3′ for the sense primer and 5′-CACGCAGCTCATTGTTGTAGAAGG-3′ for the antisense primer, giving an about 160 bp fragment. Aliquots of the DNA samples (10 μl) were loaded onto 2% agarose gel containing ethidium bromide. The PCR products were visualized with florescent illumination and measured by laser densitometer.

The results were expressed as the mean ± standard error of the mean (SEM) for the number (n) of individual experiments performed. Statistical analysis was performed using analysis of variance followed by the unpaired Student's t-test for comparisons between two groups. A probability level of $P < 0.05$ was required for statistical significance.

Three days after STZ administration, the overnight 8-h fasting serum glucose level of the diabetic rat was 398 ± 46 mg/dl, significantly higher ($P < 0.001$) than that of the control rats, 88 ± 24 mg/dl ($n = 8$ for each group). Comparisons of the body weights and bladder weights between diabetic and control rats are shown in Table 1. The diabetic bladder exhibited about four-

Table 1
Comparisons of the body weights, bladder weights, mucosal and muscle layer weights between diabetic and control rats

	Control rats	Diabetic rats
Body weight (g)	283.5 ± 13.91	307.5 ± 26.89
Whole bladder (g)**	0.10 ± 0.01	0.44 ± 0.12
Muscular layer (g)**	0.07 ± 0.02	0.30 ± 0.10
Urothelium (g)**	0.03 ± 0.01	0.14 ± 0.08
Bladder/body weight (%)**	0.04 ± 0.01	0.14 ± 0.06
Muscular layer/bladder weight (%)	71.61 ± 2.52	68.24 ± 8.24
Urothelium/bladder weight (%)	28.39 ± 8.22	31.76 ± 2.93

Values are presented as mean ± S.E.M. for $n = 8$ where n is the number of independent experiments performed.
** $P < 0.01$ for comparisons between the diabetic rats and control rats.