The Molecular Biology Study of the Neurological Mechanism of Diabetic Cystopathy in the Rat

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(可立即對外提供參考)
Abstract

The M2 receptor (M2-mAChR) is quantitatively the dominant muscarinic subtype in animal bladders. The alterations in its protein quantity and biosynthesis during diabetic cystopathy was investigated. Three-month-old male Wistar rats were divided into two groups: 1) 2-week diabetic, and 2) normoglycemic control rats. Diabetes was induced by single intravenous injection of 60mg/kg streptozotocin. The amount of M2 receptor protein in the rat bladder body tissue was measured by Western immunoblotting using monoclonal antibodies. For determination of M2 muscarinic receptor mRNA in the bladder tissue, the method of Northern blotting was employed. The results of the Western immunoblotting showed that the amount of M2-mAChR protein in the diabetic bladder was significantly increased by about 40% when compared to the control bladder (p<0.05, n=8). The Northern blotting demonstrated a 69% increase of the M2-mAChR mRNA in the diabetic bladder (p<0.05, n=8). The findings of the present study demonstrated an up-regulation of M2-mAChR biosynthesis in the diabetic urinary bladder. This phenomenon could lead to increased reactivity to acetylcholine and thus results in detrusor instability.

Keywords

Diabetes, urinary bladder, autonomic nerves, receptors
中文摘要

M2-副交感神經受體是老鼠膀胱副交感神經受體各亞型中數目最多者，其於糖尿病性膀胱病變時之變化乃本研究主要目的。三月大之雄性 Wistar 大白鼠分成二組：(1) 兩週糖尿病組；(2) 正常組。糖尿病乃以 streptozotocin 60mg/kg i.v. 誘發。膀胱中 M2-副交感神經受體 protein 含量以 Western immuno-bolting 測定；M2-副交感神經受體 m-RNA 以 Northern bolting 測定。結果發現兩者皆有明顯上升，前者約 40% (p<0.05, n=8)，後者約 69% (p<0.05, n=8)。因此可見糖尿病性膀胱病變時 M2-副交感神經受體之數量及合成皆有 up-regulation 現象。而此現象可能導致 detrusor instability 之發生。

Keywords: 糖尿病，膀胱，神經，神經受體
Introduction

Diabetic cystopathy is a common complication of diabetes mellitus. Clinically, up to 80% of patients with diabetes mellitus develop bladder dysfunction which includes decreased bladder sensation, increased residual urine or detrusor instability \[1\]. Similarly, chemically induced diabetes in rats produces bladder enlargement, failure of emptying and increased voiding frequency \[2,3\]. These disturbances are attributed to a variety of causes including axonopathy in autonomic pathways to the bladder \[4\], diuresis-induced myopathy \[3\], and metabolically induced alteration in adrenergic or cholinergic receptors in detrusor smooth muscle \[2,5\]. However the exact pathophysiological mechanism underlying diabetic cystopathy is still not clearly defined.

Streptozotocin (STZ)-induced diabetic rats have often been used as a study model for the potential mechanisms involved in the development of diabetic cystopathy. Diabetes induced by STZ treatment in rats causes profound morphological, biochemical, and pharmacological changes in the urinary bladder. Previous studies demonstrated the development of bladder hypertrophy and alterations in detrusor muscle strip responses to a variety of agonists and to electric field stimulation. Increase in the density of muscarinic cholin receptors (mAChR) in the bladder dome has been reported \[5,6\]. Our previous study has demonstrated that the vesicle-bound acetylcholine and catecholamines in the synaptosome-rich fraction of the urinary bladder were significantly increased in STZ-induced diabetic rats \[7\]. These findings suggest an impaired
neurotransmitter release mechanism in both bladder sympathetic and parasympathetic efferent nerve endings.

Muscarinic receptors have been divided into five subtypes (M1-5) according to their pharmacological characteristics. The genes for five subtypes of muscarinic receptors, m1 to m5, have been cloned [8,9,10]. It has been reported that the M2-subtype was quantitatively dominant in animal bladders [11,12]. Therefore this study aimed to define specifically the alteration of M2 receptor protein quantity and biosynthesis in diabetic cystopathy. The investigative methodologies included Western immunoblotting for M2-mAChR subtype protein and Northern blotting for M2-mAChR subtype mRNA.
Materials and Methods

Three-month-old male Wistar rats were divided into two groups: 1) 2-week diabetics, and 2) normoglycemic control rats. Diabetes was induced by single intravenous injection of 60mg/kg STZ dissolved in 0.1M citrate buffer (pH 4.5) via the lateral tail vein. The control rats were injected with the same volume of citrate buffer. Blood glucose levels were determined 72 h after STZ administration to confirm the presence of diabetes (blood glucose>300mg/dl). For obtaining bladder tissue, the rats were decapitated and bladders removed immediately via a midline abdominal incision. The bladder was immediately placed in oxygenated Tyrode's solution (NaCl 125mM, KCl 2.7mM, CaCl$_2$ 1.8mM, NaH$_2$PO$_4$ 0.4mM, MgCl$_2$ 7H$_2$O 0.5mM, NaHCO$_3$ 24mM and Dextrose 5.6mM) and the portion above the ureteral orifices was harvested as the bladder body for our studies.

The amount of M2-subtype receptor protein in the bladder body tissue was measured by Western immunoblotting using monoclonal antibodies. The bladder tissue was lysed in buffer containing 1% Triton X-100. Discontinuous slab gels (1.0mm thick) containing 0.1% SDS were prepared according to Laemmli [13] with acrylamide concentrations of 12% in the separation gel and 5% in the stacking gel. 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$_2$-subtype specific anti-mAChR antibodies (purchased from Affinity Sioreagents Inc., U.S.A.), immunostaining was performed for peroxidase activity by incubation in Tris-buffer (10mM) using enhanced chemiluminescence (ECL) development system (Amersham International,
Identification of this response was observed at 60 kDa. The observed Western immunoblots were then quantified densitometrically using a laser densitometer. The \( M_2 \)-mAChR protein concentration in the urinary bladder for the 2-week diabetic group was compared with that in the control group.

For determination of \( M_2 \) muscarinic receptor mRNA in the bladder tissue, the method of Northern blotting was employed. Total RNA was extracted from the bladder tissue according to the method of Eschenhagen (14). About 300 to 500 mg of tissue was homogenized in 8 ml ice-cold 4mol/l LiCl/8mol/l urea with a polytron. Incubation was performed overnight at 4°C. Total cellular RNA was precipitated by centrifugation at 17,000 g for 30 min at 0°C. After proteinase K (Boehringer Mannheim, Mannheim, FRG) digestion in 0.01mol/l Tris, 0.5% SDS, pH 7.5 at 37°C for 2 h followed by three phenol/chloroform extractions, the RNA was precipitated with 0.2mol/l sodium acetate and 70% ethanol overnight, followed by centrifugation at 10,000 g for 30 min at -10°C. The RNA pellet was dissolved in 10 mmol/l Tris, 1 mmol/l EDTA, 0.5% SDS, pH 7.8.

RNA blotting was performed as followed. Aliquots (15-20ug) of RNA were denatured with 42% formamide, 5.8% of formaldehyde at 95°C for 2 min and size-fractionated by electrophoresis in 1% agarose gels containing 2% formaldehyde and 0.5ug/ml ethidium bromide (Fluke Chemie, Buchs, Switzerland). RNA was transferred to Hybond N nylon membrane (Amersham, Braunschweig, FRG) by Northern blot capillary transfer overnight using 20 x SSC as transfer medium.

For preparation of cDNA probes, plasmid DNA containing \( M_2 \) subtype inserts propagated in E. coli was isolated, linearized and fragmented with RsaI. A 1.4 kb DNA fragment containing the insert was isolated by gel electrophoresis
and nick translated with $^{33}$P-dCTP.

Blot membranes were prehybridized at 42°C overnight in a solution containing: 50% formamide, 5 x Denhardt (Ficoll, polyvinylpyrrolidone and BSA, 1 mg/ml each), 0.9mol/l NaCl, 0.06mol/l NaH$_2$PO$_4$, 0.006mol/l EDTA, 0.1% SDS, 200ug/ml tRNA from yeast. Radiolabelled probes were added to fresh hybridization solution at a concentration of 1-2x10$^6$ dpm/ml. Hybridization was performed at 50ug/cm$^2$, 42°C for 48 hr. After hybridization, blot membranes were washed with 2 x SSC, 0.1% SDS, followed by 10-min incubation in 2 x SSC, 0.1% SDS at room temperature and three 20-min washes in 0.2 x SSC, 0.1% SDS at 65°C. Wet blot membranes were sealed in plastic foil and exposed to medium-sensitive medical X-ray film (R2, 3M, Italy) at -80°C using intensifier screens. Exposition times were 3-14 days. Autoradiographyic densities of the M$_2$ bands were compared with GAPDH which was used as the standard for comparisons. The M$_2$-mAChR mRNA concentrations in bladder tissue of the diabetic and control rats were compared.

The results were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using analysis of variance followed by the Students t test for comparisons between two groups. A probability level of <0.05 was required for statistical significance.

**Results**

The result in figure 1 showed the Western blotting for bladder M$_2$-mAChR protein. With increasing loading protein concentrations, the amount of M$_2$-mAChR protein also showed dose-dependent increases. Evidently, the amount of M$_2$-mAChR protein in the diabetic bladder was increased compared
to the control bladder. Quantitative presentation of the result was shown in figure 2. The bladder M₂-mACHR protein increased about 40% in the 2-week diabetic rats.

Figure 3 showed a result from the Northern blotting for bladder M₂-mACHR mRNA which presented as a 5.6 kb band. The quantitative comparison using GAPDH as internal standard was given in figure 4, which demonstrated a significant 69% increase of the mRNA in the diabetic bladder.
Discussions

The results of the present study demonstrated early in the course of STZ-induced rat diabetes, there is an up-regulation of bladder \( M_2 \)-mAChR biosynthesis; thus explaining, at least partially, the long observed phenomenon of increase in muscarinic receptor density in diabetic bladders. Clinically, urodynamic findings in diabetic cystopathy often showed impaired bladder sensation, increased cystometric capacity, decreased bladder contractility, impaired uroflow, and residual urine. Cystometric examination may show detrusor areflexia, which is usually found in patients with impaired sensation of bladder filling, evidence of sensory neuropathy. On the other hand, detrusor hyperreflexia (instability) has also been found in diabetic patients and Hochberg et al. found this to be the more likely finding than detrusor areflexia in 44 adult-onset diabetics with varied voiding symptoms [15]. Theoretically, our present finding of increased \( M_2 \)-mAChR biosynthesis and quantity in the diabetic bladder may lead to de-stabilization of the urinary bladder as it would become more sensitive to the released neurotransmitter acetylcholine. Therefore, the \( M_2 \)-mAChR up-regulation is one possible explanation for the occurrence of detrusor hyperreflexia in diabetics. However, since generalized neuropathy is a common complication during diabetes, dysfunction of the cortical or spinal regulatory tracts for the urinary bladder must also be considered.

The mechanism leading to the up-regulation of \( M_2 \)-mAChR needs further investigation. By studying synaptosome-rich fractions prepared from STZ-induced diabetic rat bladder, it was demonstrated that the vesicle-bound neuronal acetylcholine increased by 4-fold [7]. In 3-month alloxan-induced diabetic rats, it was demonstrated that the number of myelinated fibers in the
pelvic and hypogastric nerves was greatly reduced. Large- and medium-sized axons were rare or absent while signs of de- and re-myelination were present [16]. It has also been shown that 9 weeks after STZ treatment, the mean cross-sectional area for retrogradely labelled bladder neurons in the major pelvic ganglion was greater in diabetic than in control rats. The amount of vasoactive intestinal polypeptide (VIP), a co-transmitter of acetylcholine, was increased four-fold in the diabetic bladder [4]. In view of the aforementioned evidence, it has been postulated that an axonopathy-related neurotransmitter release defect leads to an accumulation of neuronal neurotransmitters including acetylcholine. Such a functional denervational status eventually leads to an up-regulation of the post-synaptic muscarinic receptors [7].

Pharmacologically, muscarinic receptor subtypes M1, M2 and M3 have been defined according to their affinities for pirenzepine and AF-DX 116 [17, 18]. Moreover, the genes for five subtypes of muscarinic receptors, m1-5 have been cloned [8]. Antagonist inhibition studies on isolated human urinary bladder has shown a lack of M1 receptors in the detrusor muscle [19]. Studies have demonstrated the presence of both M2 and M3 receptors in human as well as animal bladders with the former being quantitatively predominant, about 85% in the rat urinary bladder [20, 21]. Functionally, it was found that in spite of its minority, M3 subtype is important in mediating detrusor muscle contraction. Activation of M3 receptors via the G protein Gq results in increased polyphosphoinositide hydrolysis, release of Ca\(^{2+}\) ions from the sarcoplasmic reticulum and consequent muscle contraction [11, 12]. The physiological role of M2 receptors has yet to be defined. Recently, it has been suggested that the bladder M2 receptors are responsible for causing indirect contraction of the
detrusor muscle by reversing sympathetically mediated relaxation [22].

Beta-adrenoceptors, particularly \( \beta_{3} \)-adrenoceptors, have been implicated to exert a sympathoinhibitory drive to maintain bladder relaxation during urine storage. It has been postulated that during voiding, acetylcholine released from postganglionic parasympathetic nerves, activates \( M_2 \) receptors to reverse the sympathoinhibitory tone and this, in concert with direct \( M_3 \) receptor detrusor contraction, allows more efficient and complete voiding of urine. Thus the diabetes induced up-regulation of bladder \( M_2 \) mAChR theoretically can topple detrusor stability by undermining the sympathoinhibitory effect during bladder storage.

In conclusion, the findings of the present study demonstrated the up-regulation in quantity and biosynthesis of \( M_2 \) mAChR in the urinary bladder of 2-week STZ-induced diabetic rats. These observations offer an explanation to the well-known phenomena of increased reactivity to acetylcholine and muscarinic receptor bindings in diabetic urinary bladders. Theoretically, the up-regulation can lead to reduction of the sympathoinhibitory tone and results in detrusor instability.
References

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Figure 1. Autoradiograph of Western blotting for bladder M2-mAChR protein (lanes 1, 3, 5: Wistar rat bladder loading protein 100, 200, 300 μg respectively; lanes 2, 4, 6: diabetic rat bladder loading protein 100, 200, 300 μg respectively). The amount of M2-mAChR protein in the diabetic bladder was increased compared to the control bladder.
Figure 2. Comparisons of the bladder M2-mAChR protein amount between diabetic and control Wistar rats. Western blotting was performed with loading protein 100, 200 and 300 – g respectively. The value for Wistar rat bladder with loading protein 100 – g was taken as 100%.

(*p<0.05, **p<0.01 for n=8)
Figure 3. Autoradiograph of the Northern blotting for bladder M2-mACHR mRNA (lanes 1,3,5: Wistar rat bladder with loading RNA 15, 25 and 35 μg respectively; lanes 2,4,6: diabetic rat bladder with loading RNA 15, 25, 35 μg respectively). The mRNA was presented as a 5.6 kb band. GAPDH was used as internal standard.
**Figure 4.** Comparison of the bladder M2-mAChR mRNA amount between diabetic and control Wistar rats. Values were calculated from Northern blotting with 15-μg loading RNA and GAPDH as the internal standard. (*p<0.05, **p<0.01 for n=8)