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The function of hyperpolarization-activated cyclic nucleotide-gated channel in diabetic cystopathy

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Abstract. – OBJECTIVE: We aimed at investigating changes in the expression and physiological function of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in interstitial cells of Cajal (ICC) in diabetic state.

MATERIALS AND METHODS: Twenty adult female Sprague-Dawley (SD) rats were randomly assigned to control and Zucker diabetic fatty (ZDF) group. The protein and mRNA expression of HCN isoforms and C-kit in the rat bladders were detected using Western blotting and reverse transcription-polymerase chain reaction (RT-PCR). The bladder contraction was evaluated using a bladder smooth muscle strip test. Whole cell patch-clamp techniques were used to detect the activity of HCN channels. Immunofluorescent staining was used to the positive expression of HCN and C-kit in ICC.

RESULTS: cAMP, as HCN channel-specific stimulant, could increase the frequency and amplitude of spontaneous contractions in both group, while cAMP inducing contraction of ZDF rats, was still significantly lower compared with the control group. Acute bladder ICCs were isolated by collagenase digestion. Classic Ih current pattern was recorded on ICCs while Ih current amplitude of ICCs from ZDF diabetic rats was significantly lower than the control group. The expression and mRNA of HCN1-4 isoforms in ZDF diabetic rats were both significantly lower compared with the control group. Meanwhile, the number of c-kit positive cells in ZDF diabetic rats showed no significant differences compared with controls. The morphological structure of ICC in the bladder of ZDF rats was relatively loose and the number of their cell process was apparently decreased.

CONCLUSIONS: The structure of ICCs in ZDF rats was relatively loose, their connection to each other was also diminished. The expression of HCN was down-regulated and its response to cAMP was also decreased. HCN channels in bladder ICCs might regulate detrusor contraction. Changes in HCN expression and activity in bladder ICCs might be one of the most important mechanisms of diabetic cystopathy.

Key Words:
Hyperpolarization-activated cyclic nucleotide-gated, Zucker diabetic fatty, Interstitial cells of Cajal, C-kit, cAMP.

Introduction

Diabetic cystopathy (DCP) is a diabetes-induced dysfunction in the bladder and urinary tract. DCP is one of the most common complications of diabetes whose morbidity is 25-80% in diabetes patients[1,2]. DCP reduce bladder sensation and contractile function in varying degree[3]. In some worse conditions, even upper urinary tract function is impaired[4]. DCP badly affects the quality of life of diabetes patients[5]. Pathogenesis of DCP has not been fully clarified. Then, it is believed that peripheral neuropathy induced by high glucose condition was the only cause of DCP[6]. Increasing evidence provided by recent researches indicated that the myogenic factor of bladder detrusor itself played an indispensable role in the pathogenesis of DCP[7-9]. Smet et al[10] provided the first evidence that bladder might contain the interstitial cells of Cajal (ICC) by observing cyclic guanosine monophosphate-immunopositive cells in guinea-pig and human bladders, which had a morphological resemblance to gut ICC, and they existed between tunica submucosa and smooth muscle of the bladder. In the structure of tissues, bladder ICC are closely connected with neural cells and detrusor cells[11,12]. As for function, resembling gut ICC, bladder ICC serve as pacemaker cells and play important roles in controlling bladder excitability. Further investigation[13] suggested that hyperpolarization-activated cyclic nucleotide-gated (HCN) channels were specific channels of pacemaker cells, which were believed to conduct an inward hyperpolarization-activated cation current (I_h) to participate

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in physiological events such as pace-making and excitability in heart, brain and bladder. HCN1-4 isoforms were expressed by ICC, which could generate I_h [14-16]. In various morbid states, such as bladder outlet obstruction (BOO), changes in activation and physiological function of HCN would lead to dysfunction of bladder excitability[17-19]. Changes in expression and activity of HCN in the diabetic state were seldom reported. This research aimed at exploring changes in expression and physiological function of HCN in ICC in diabetic state. In this work, urodynamics characteristics of Zucker diabetic fatty rats were observed, expression and activity of c-kit and HCN were detected, immunofluorescence staining was applied to ICC and contractile experiment of isolated smooth muscle strips was also conducted.

Materials and Methods

Animals

Zucker diabetic fatty (ZDF) rats (n=10, weigh 250-280 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China). Normal Sprague-Dawley (SD) rats (n=10, weigh 250-280 g) were set as a control group. ZDF rats were treated with high-glucose-high-fat diet. Fasting blood-glucose of rats in each group was detected. Rats with a fasting blood-glucose higher than 16.7mmol/L were selected. The Ethics Committee approval was granted by our Hospital.

Measurement of Post-Void Residual Urine Volume and Bladder Pressure

After anesthetized by Pentobarbitol, ZDF rats and rats in the control group were fastened to operation panel. The bladder of rats was exposed by a median incision of lower abdomen. An epidural catheter was implanted in the bladder through the body of the bladder; after the catheter was properly fixed, an urodynamometer and a microinfusion pump were connected *via* a three-limb tube. Zero point was set. Saline was used to fill the bladder with a fill rate of 0.2 ml/min. Change of bladder pressure and volume was recorded by a multiple channel bio-signal collecting systems during this filling process, which ended when urination was finished. The bladder was lightly pressed and the volume of residual perfusate was recorded as post-void residual urine volume. At least three urination cycles were recorded for each rat. Maximum bladder volume, maximum bladder pressure and post-void residual urine volume were recorded.

Contractile Experiment of Isolated Smooth Muscle Strips

30 min after all measurement of filling bladder pressure was finished, the bladders of rats in different groups were collected and stored in Krebs solution at 4°C. Every bladder was sliced into 10 mm × 2 mm detrusor strips. Both ends of detrusor strip were tied by silk threads, and the lower ends were fixed in the bottom of an organ bath. Strips were entirely immersed in Krebs solution at 37°C. The upper end was fixed to a trimming bolt, which could regulate the length of the strip, through a tension sensor. A bio-signal recorder was used to collect information from the sensor. 95% O_2 and 5% CO_2 were blown into Krebs solution and the detrusor strips were stabilized for 30 min. Strips were pulled lightly until the tension reached an initial value of 0.75 g. Frequency and amplitude of spontaneous contractions were recorded by a RM6280 physiological signal collecting and handling system. Changes in frequency and amplitude were observed after exogenous cAMP (100 μM) was added.

Immunofluorescence Staining of ICC

5 ZDF rats and 5 SD rats were randomly picked, and their bladders were collected after being executed by cervical dislocation. Both ureters were ligated, and an epidural catheter was inserted *via* the urethra. 4% paraformaldehyde was used to fill bladders, and all bladders were immersed in 4% paraformaldehyde at room temperature overnight. After tunica serosa, tunica mucosa of bladder tissue was removed and tissue stretched preparation was made under the microscope, immunofluorescence staining of c-kit and HCN channel protein were applied. The number and morphological characteristics of cells with double staining were observed under a confocal laser-scanning microscope. 20 fields (×100) of each group were selected for calculating the number of ICC.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted by utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed to generate the first-strand cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). mRNA expression was detected with SYBR Premix Ex Taq II (TaKaRa, Dalian, China) through qRT-PCR analyses. The total RNA was reverse-transcribed with a miScript reverse transcription kit (Qiagen, Hilden, Germany) and then amplified with SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) to quantify the miRs. $2^{-\Delta\Delta CT}$ method was

applied to determine the relative gene expression, and the expression levels of HCN1-4 and C-kit were normalized to GAPDH and U6, respectively. PCR amplification was repeated three times for each gene.

Western blotting

Total protein was extracted from the tissues by using a radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China). The lysates were obtained using RIPA buffer containing a protease/phosphatase inhibitor mixture (diluted 1:100, Vazyme Biotech, Nanjing, China) and then subjected to 10000 g centrifugation at 4°C for 20 min. Total protein concentrations in the supernatant were determined by bicinchoninic acid assay (Beyotime Biotechnology, Shanghai, China). A total of 30 μg protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by electroblotting. After blocking with 5% bovine serum albumin (BSA) at room temperature for 120 min, the blots were incubated with primary antibodies overnight at 4°C and developed with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies (diluted 1:2000, Beijing Zhongshan Biotech Company, Beijing, China) at room temperature for 60 min. Enhanced chemiluminescence (ECL) kit (Beyotime Biotechnology, Shanghai, China) was used to produce chemiluminescent signals, which were recorded by a Chemi Doc™ XRS Imaging System (Bio-Rad, Hercules, CA, USA). Band intensity was quantitated using Image Pro-Plus software. Protein expression levels were represented as densitometric ratios of the targeted protein relative to GAPDH.

Detection of Activity of c-kit and HCN Channels

Whole-cell patch clamp technique was applied to record the current generated by bladder ICC. Holding potential (HP) = -60 mV, sampling frequency = 2 kHZ. The whole experiment was conducted at room temperature (18-26°C). The given HP to ICC was set at -60 mV. From -60 mV to -120 mV, voltage stimulation was given by a gaining step of -10 mV. The stimulation time was 800 ms and the stimulation interval was 1 s. The graph obtained using patch clamp resembled inward hyperpolarization-activated cation current generated by HCN channels.

Statistical Analysis

Data were statistically analyzed by SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Data from three independent experiments were presented as mean ± standard deviation (SD). One-way ANOVA among groups and two-tailed Student's *t*-test were performed to compare between groups. $p < 0.05$ was considered statistically significant.

Results

ZDF Rats Model and Pressure of Filling Bladder

Fasting blood-glucose of 2 months old ZDF rats was 36.4±5.6 mmol/L, which was significantly higher than that of SD rats 5.1±0.4 mmol/L ($p < 0.05$). The result of pressure measurement of filling bladder was depicted in Table I. Compared with control group, the maximum bladder volume and post-void residual urine volume of ZDF rats were significantly increased, while the maximum bladder pressure was significantly decreased ($p < 0.05$).

Contractile Experiment of Isolated Smooth Muscle Strips

Detrusor strips of bladders from ZDF rats and SD rats were lightly pulled to induce spontaneous contraction. When an initial tension (0.75 g) was imposed, frequency and amplitude of spontaneous contractions of ZDF rats were both significantly lower than rats from the con-

Table I. The pressure of filling bladder in two groups.

Group	Maximum bladder volume (ml)	Maximum bladder pressure (cmH_2O)	Post-void residual urine volume (ml)
ZDF	3.53±0.42*	34.65±6.32*	1.54±0.76*
Control	1.89±0.31	75.78±12.70	0.12±0.03

*Compared with control, $p < 0.05$.

Table II. Contractile experiment of isolated smooth muscle strips.

	Spontaneous contraction		cAMP induced contraction	
Group	**Amplitude (g)**	**Frequency (Hz)**	**Amplitude (g)**	**Frequency (Hz)**
ZDF	0.15±0.06*	0.09±0.02*	0.26±0.11*#	0.12±0.04*#
Control	0.77±0.08	0.18±0.05	1.13±0.21#	0.28±0.07#

trol group (p<0.05) (Table II). When exogenous cAMP was added into the system as a HCN channel-specific stimulant, the frequency and amplitude of spontaneous contractions in both groups were significantly increased (p <0.05), while cAMP induced contractions of ZDF rats were still significantly lower compared with the control group (p <0.05) (Table II). Frequency and amplitude of spontaneous contractions of ZDF rats were both significantly lower than SD rats; cAMP could increase contraction ability of detrusor strips, but cAMP induced contraction of ZDF rats was still significantly lower compared with the control group.

The Activity of HCN Channels

HCN channel-specific inward hyperpolarization-activated cation current could be detected in bladder ICCs of ZDF rats and SD rats (Figure 1A and B). I_h was activated at -90 mV, which could be observed from -90 mV to -120 mV. The current amplitude was about 50 pA, and it was recorded at -120 mV. Interestingly, the current amplitude of I_h in ZDF rats group was significantly lower compared with the control group (p<0.05), which indicated that HCN function in bladder ICC of ZDF was possibly impaired.

The Expression Level of c-Kit and HCN1-4 Isoforms in Bladder

The protein expression of c-kit and HCN1-4 isoforms in bladder of ZDF rats was significantly lower compared with SD rats (p<0.05) (Figure 2A and B) and the mRNA expression of c-kit and HCN1-4 isoforms in bladder of ZDF rats was signficantly lower compared with SD rats (p<0.05) (Figure 2C), indicating that the expression of c-kit and HCN channels was decreased in bladder ICC of ZDF rats.

The Positive Expression of HCN and c-Kit in ICC

Immunofluorescent staining was used to detect the positive expression of HCN and C-kit in ICC. In ZDF rats, the number of c-kit positive and HCN channel protein positive cells (10.3±5.8) were decreased compared with control group (13.2±7.5), though no significant difference was found (Figure 3A and B). It was found that morphological structure of ICC in

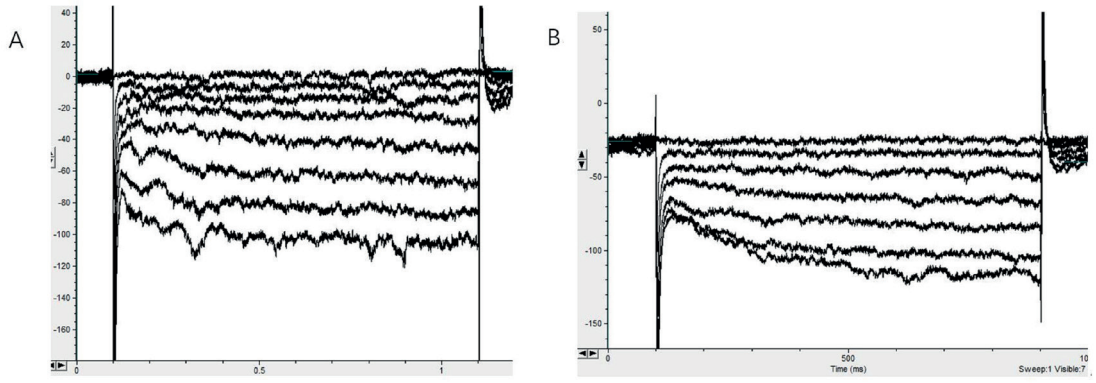

Figure 1. Ih current density and Ih activation curves in the ICCs. ***A,*** Current responses evoked by hyperpolarizing voltage steps from -60 to -120 mV in ICCs from control group was detected by whole cell patch-clamp techniques. ***B,*** Current responses evoked by hyperpolarizing voltage steps from -60 to -120 mV in ICCs from ZDF group was detected by whole cell patch-clamp techniques.

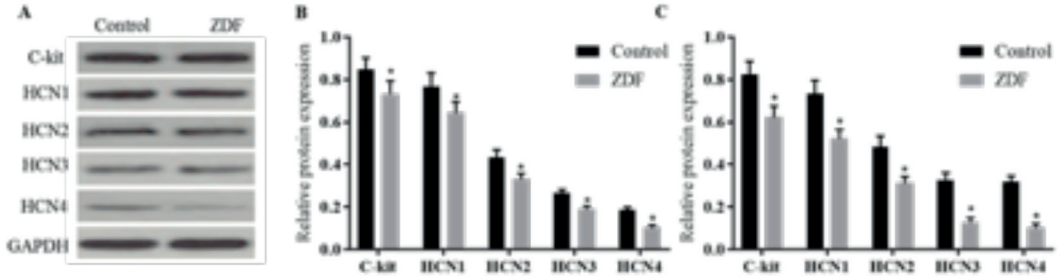

Figure 2. The expression level of c-kit and HCN1-4 isoforms in bladder. ***A-B,*** The protein expression level of c-kit and HCN1-4 isoforms in bladder were detected by Western blotting. ***C,*** The mRNA expression level of c-kit and HCN1-4 isoforms in bladder were detected by PCR. Data are shown as mean ± SD based on at least three independent experiments. ** $p < 0.01$ by Student's *t*-test.

bladder of ZDF rats was loose and the number of their cell process was decreased. The connection between cells and the connection between smooth muscle were relatively fewer.

Discussion

The bladder is a hollow viscera mainly consisting of smooth muscle, whose mechanism of storing and eliminating urine has not been fully clarified[20]. It has been a very attractive topic to investigate the pathogenesis of urination disorder[21]. The discovery of bladder ICC with spontaneous electrical activity lays a very important foundation for the myogenic theory of regulation of bladder excitability[22]. Bladder ICCs are mainly distributed alongside smooth muscle and closely connected with smooth muscles and nerves[23,24]. Neurostimulation can induce spontaneous activity of bladder ICC. Under mechanical stretch stimulation, spontaneous calcium activity in ICC is increased, and ICCs transmit the impulse signal to smooth muscles. Those networks of ICCs are widely distributed in the bladder, which form functional complexes with smooth muscle cells and nerve cells and transmit information *via* gap junction[25,26]. Those characteristics remind us that bladder ICC can possibly serve as a regulating bladder excitability and mobility. According to former researches of our institution, changes were observed

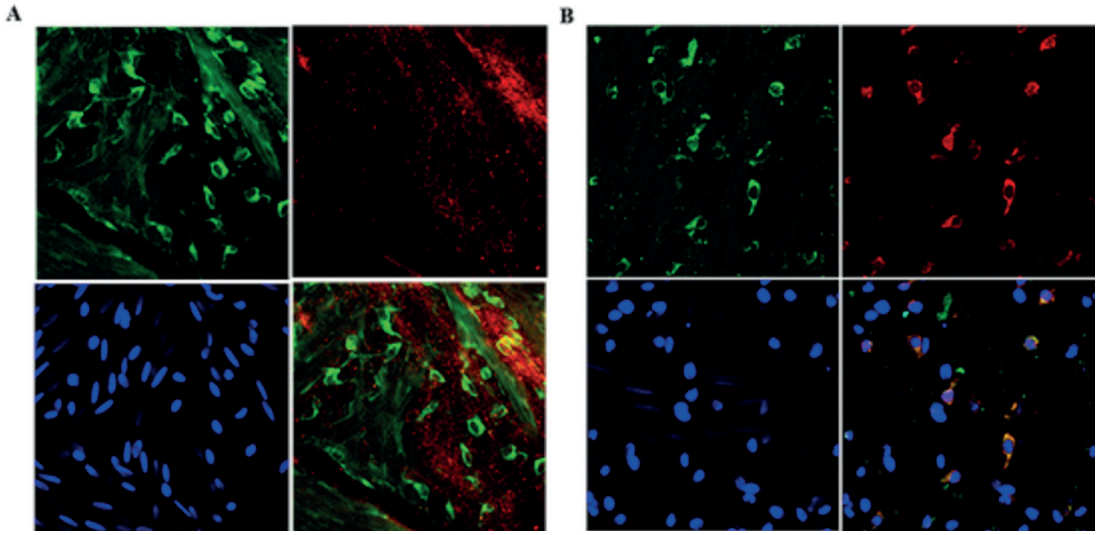

Figure 3. The positive expression of HCN and C-kit in ICC. ***A,*** The positive expression of HCN and C-kit in ICC in control group was detected by immunofluorescence staining. ***B,*** The positive expression of HCN and C-kit in ICC in ZDF group was detected by immunofluorescent staining. Green represents C-kit, red represents HCN and blue represents cell nucleus.

in ICCs from bladders with detrusor overactivity or detrusor inactivity[27,28]. In morbid states such as neurogenic detrusor overactivity and diabetic cystopathy, the special, net-distributed bladder ICCs suffered an enormous change in number, distribution density, and structure. The cellular morphology and ultrastructure of ICC were significantly altered compared with ICC in a normal state. When ICC function was blocked, the contraction of detrusor was also suppressed. The abnormality of ICC function might be the central link that regulated bladder excitability in diabetic cystopathy.

Among many driving force sources of excitable cell, the mechanism of ion channels is deeply and widely investigated. Resembling interstitial cells of Cajal, net-distributed bladder ICCs integrate, transmit signals, and are spontaneous excitatory[29,30]. HCN channels are closely related to the autorhythmic ability of ICCs, which became a focus in our research[31,32]. HCN channels are a group of proteins which are widely believed as pacemaker channels. They can be activated in the process of cell hyperpolarization and generate inward current which is called current I_h. Activated HCN channels in the process of hyperpolarization awake the cell and participate in the initiation of cell excitation[33,34]. HCN channels not only play important roles in regulating rhythmic activity and spontaneous electrical activity of myocardial cells and nerve cells. Indeed, they are also related to many basic physiological events such as heartbeat, respiration, sleep and the secretion and release of neurotransmitter. HCN channels are crucial to maintain electrophysiological characteristics of various kinds of somatic cells[35,36].

Conclusions

In the bladder, HCN channels regulate bladder excitability *via* modulation of ICC spontaneous excitability. Our investigation showed that the structure of ICCs in ZDF rats was relatively loose, and their connection to each other was also diminished. The expression of HCN was downregulated and its response to cAMP was also decreased. The mentioned evidence indicated that HCN channels in bladder ICCs might regulate detrusor contraction. Changes in HCN expression and activity in bladder ICCs might be one of the most important mechanisms of diabetic cystopathy. Researches in HCN channels and their regulating factor can be treated as a cutting point to clarify the mechanism of abnormal bladder contraction and excitability and to enrich urinary physiology theories. This is very hopeful to throw light upon an investigation into the pathogenesis of diabetic cystopathy and to give a novel target for clinical diagnosis and treatment.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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