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Original Article

Effect of glucose concentration on the expression of HSP27, CaD and TM proteins in rat detrusor smooth muscle cells

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Abstract: Objective: To investigate the effect of glucose concentration on the expression of proteins related to the contraction of rat detrusor smooth muscle cells (DSMCs). Methods: DSMCs were cultured and isolated in vitro. The expression of HSP27, CaD and TM genes and proteins were detected by fluorescence immunoassay, RT-PCR and Western Blot, respectively. Results: Compared with the control group, the expression of CaD and TM in the experimental groups increased significantly, while the expression of HSP27 decreased. Conclusion: The diabetic bladder dysfunction model was built in rat DSMCs.

Keywords: Glucose, rat detrusor smooth muscle cell

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder, whose incidence has been increasing due to the rising of people's living standard, population ageing and the change of life style [1]. Diabetic bladder dysfunction (DBD) is a common complication of DM [2], occurring in 40%-100% of DM patients. Even among those with an effective control of blood glucose, the incidence of DBD is still as high as 25% [3]. DBD is featured by hidden onset in early stage and usually asymptomatic progressive development [4]. Therefore, DBD is easily ignored until the appearance of chronic urinary retention and urinary retention with overflow incontinence due to DBD. At this time, the patients may suffer greatly from reflux hydronephrosis and renal function impairment due to irreversibility of DBD and secondary urinary tract infection [5]. Hence it is important to protect detrusor smooth muscle cells (DSMCs) and to promote its recovery. We built the DBD model in rat DSMCs and studied the expressions of heat shock proteins 27 (HSP27), caldesmon (CaD) and tropomyosin (TM), the proteins related to contraction of DSMCs, under different glucose concentrations. We hope to find new clues for understanding the pathogenesis of DBD, preventing contractile dysfunction of DSMCs, identifying target molecules and evaluating prognosis.

Materials and methods

Animals

SPF SD rats weighing 200-220 g were purchased from Experimental Animal Center of Third Military Medical University.

Reagents

DMEM-F12 and fetal bovine serum (Gibco, USA), penicillin and streptomycin (Beyotime, China), glucose (Sigma, USA), HSP antibody (rabbit, self-prepared), specific phosphorylated HSP27 antibodies (hsp27-ser15, hsp27-ser78, hsp27-ser82, self-prepared), TM antibody (mouse, self-prepared), CaD antibody (rabbit, self-prepared), anti-rabbit 647 secondary antibody (RD Corporation, USA). Primers, RT-PCR reagents and fluorescent quantitative PCR reagents were purchased from Western Biotechnology (USA).

Table 1. Primers used for real-time fluorescent quantitative PCR

Gene	Name	Sequent	Length	Source
CaD	rCaDF	TGGTCTCCAAGATTGACAGCC	135 bp	The present study
	rCaDR	ACATGCTCTTGATATTGCGGAC		
TM	rTMF	AAGTCATTGAAAGCCGAGCC	190 bp	The present study
	rTMR	GCACATTTGCCTTCCGAGA		
B-actin	rat actin f	CCCATCTATGAGGGTTACGC	150 bp	Literature [8]
	rat actin r	TTTAATGTCACGCACGATTTC		

Primary culture and identification of rat DSMCs

The rats were sacrificed by cervical dislocation and the abdomen was opened on the ultra-clean bench. The bladder was harvested, and the mucosal layer was removed. The tissue was cut into pieces and placed into the centrifuge tube containing 10 ml of 0.1% collagenase P. The cells were cultured at 37°C for 45 min to 1 h. After washing, the cells were resuspended in DMEM/F12 containing 15% FBS and placed into the CO_2 incubator. The cell adherence to the wall was observed and the culture medium was replaced every two days. The cells climbed out from the tissue mass about 2 days later. The fused cells were digested 1 week later. Immunohistochemical identification of the isolated cells was performed using rabbit α-SM-actin antibody. After sealing at room temperature with goat serum for 60 min, the cells were incubated with primary antibody (1:500) at 4°C overnight. The cells were washed with PBS and incubated with biotinylated secondary antibody at 37°C for 30 min. Alkaline phosphatase-conjugated streptavidin working solution (wet box) was added to incubate the cells at 37°C for 30 min. DAB reagent was added for color development, followed by negative staining, gradient dehydration, transparentization in xylene, slide sealing and microscopy.

Building of rat DSMC model under high glucose

Log phase DSMCs were harvested, digested, centrifuged and coated onto the plate. After the cells were cultured for 24 h, the old culture medium was replaced and DMEM-F12 containing glucose of different concentration was added into each well. For the control group, 5 mM glucose was added, and for the high glucose group, 50 mM glucose was added. The cells were further cultured in the incubator for 48 h. The cells were observed under the inverted microscope.

Detection of HSP27 expression and phosphorylation level in DSMCs under different concentrations of glucose by fluorescence immunoassay

The method was based on the literature [6]. The cells were fixed in paraformaldehyde, dehydrated, transparentized and embedded and subjected to antigen hot repair. The slides were washed with PBS, sealed with 6% goat serum for 1 h and incubated with specific phosphorylated HSP27 primary antibody (1:200) at 4°C overnight. Then the cells were incubated with 647 secondary antibody (1:1000) at 37°C for 30 min. The cells were stained with DAPI in the dark for 15 min. The images were observed by the laser scanning confocal microscope.

Detection of expression of CaD and TM by QRT-PCR

By reference to the literature [7], total RNA was extracted using Trizol, chloroform, isopropyl alcohol and ethanol. For the control group and the experimental groups, 3 replicates were set up, respectively. The extracted RNA was reversely transcribed into cDNA using random primers. Fluorescent quantitative PCR was carried out using specific primers of CaD and TM and sybr green I dye in **Table 1**, with rat β-actin as the internal reference. The system of reverse transcription was as follows (20 ul): 2 × RT buffer 10 ul, 6N random primers (100 pmol/ul) 1 ul, RT mix 1 ul, template 5 ul and DEPC-treated water 3 ul. Reaction conditions: 25°C for 10 min, 42°C for 50 min, and 85°C for 5min. The system of fluorescent quantitative PCR (50 ul) was as follows: 2 × PCR buffer 25 ul, primers (25 pmol/ul) 1 ul × 2, Sybr green I (20 ×) 0.5 ul, template 2 ul, DEPC-treated water 20.5 ul. Reaction conditions: 94°C for 4 min, 94°C for 20 s, 60°C for 30 s, 72°C for 30 s, 35 cycles, final extension at 72°C.

The relative copy number of each gene was calculated according to the standard curve. Its ratio to the relative copy number of the internal reference was taken as the corrected value of mRNA content of each gene. The experimental

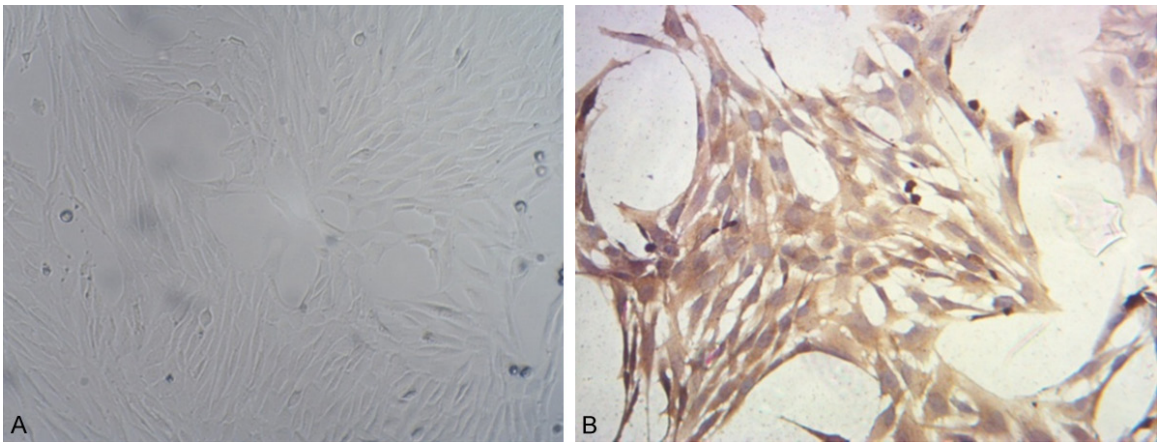

Figure 1. Morphology of rat DSMCs in primary culture and immunohistochemical identification of DSMCs. A. Rat DSMCs after passage; B. Immunohistochemical identification of actin.

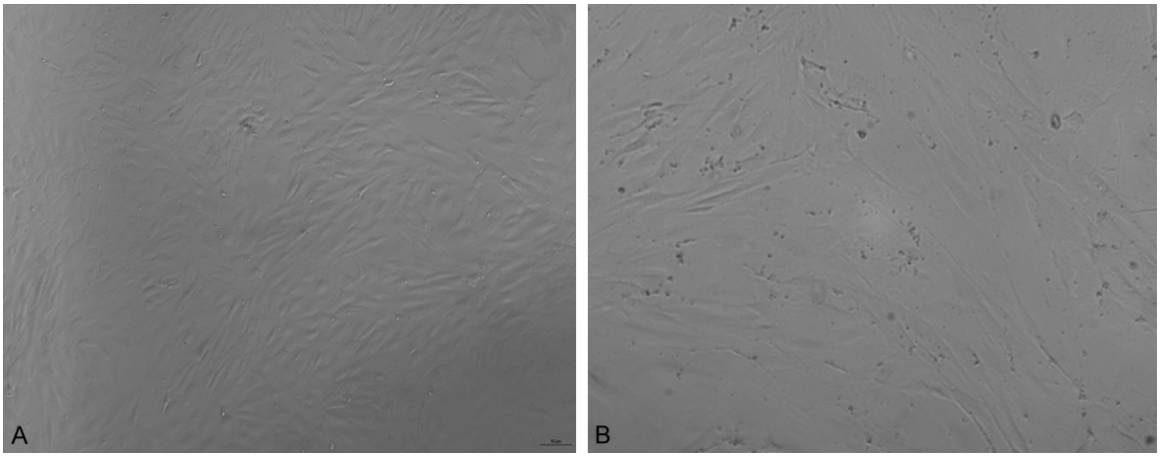

Figure 2. Morphology of DSMCS under different concentrations of glucose. A. Control group (5 mM glucose); B. Experimental group (50 mM glucose).

groups and the control group were compared on this basis. t-test was performed pairwise.

Detection of the expression of HSP27, specific phosphorylated HSP27, CaD and TM by WB

Referring to the literature [9], 3 replicates were set up for each group. The extracted total proteins were analyzed by 4% SDS-PAGE, transferred to membrane and sealed in TBST buffer containing 5% defatted milk powder for 1 h. The membrane was incubated with diluted primary antibodies (1:500, HSP, hsp 27-ser15, hsp27-ser78, hsp27-ser82, CaD, TM) and GAPDH primary antibody (glyceraldehyde-3-phosphate dehydrogenase, 1:1000) at 4°C overnight. The cells were washed and added with HRP-conjugated secondary antibody (1:2000) for 1.5 h. The protein content of the band was determined using enhanced chemiluminescence (ECL) detection system Amersham Biosciences.

Results

Morphology of rat DSMCs in primary culture and immunohistochemical identification of DSMCs

After cell passaging, the rat DSMCs presented the shape of short rod (**Figure 1A**). The result of immunohistochemical identification is shown in **Figure 1B**.

Building of rat DSMC model under high glucose

Microscopy result is shown in **Figure 2**. The morphology of DSMCs did not show obvious

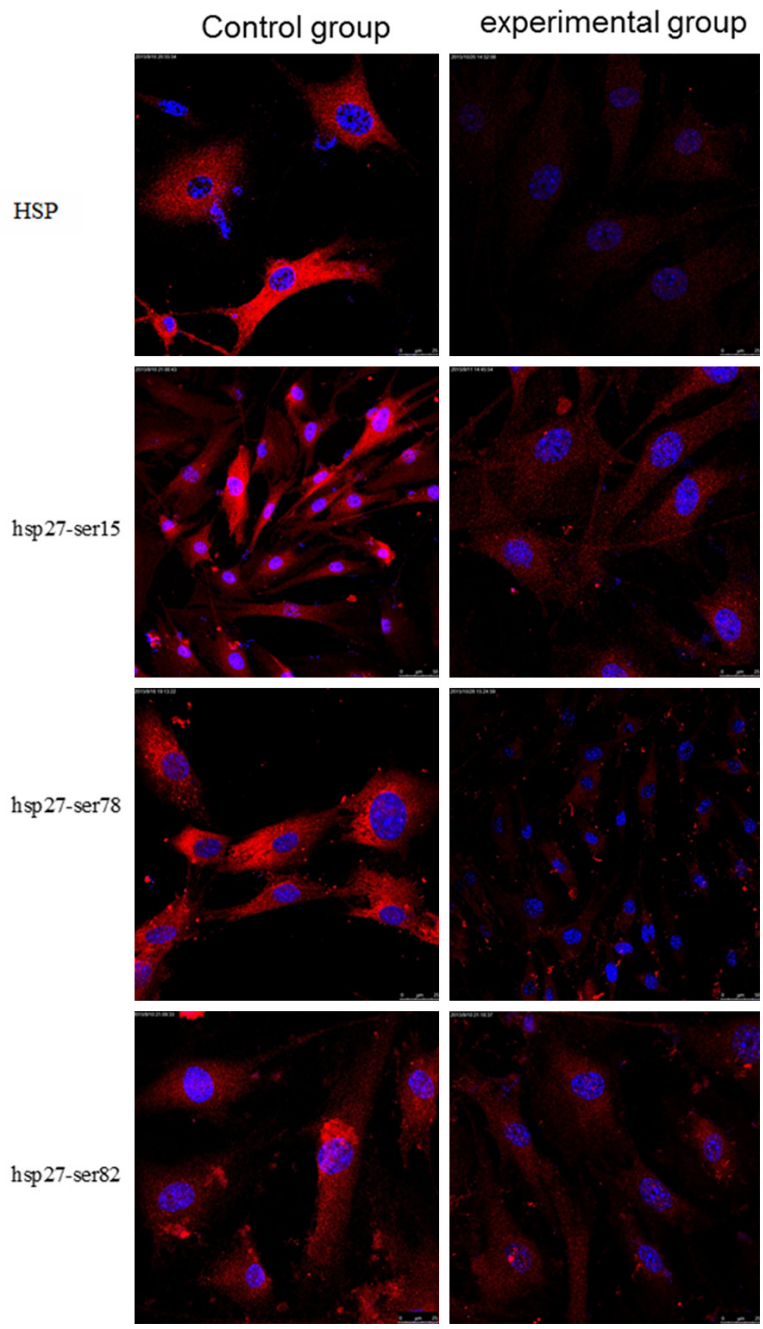

Figure 3. Expressions of HSP27 and phosphorylated HSP27 under different concentrations of glucose by fluorescence immunoassay.

variations under different concentrations of glucose, except for mild shrinkage of a few cells.

Expression of HSP27 and the phosphorylation level under different concentrations of glucose by fluorescence immunoassay

As shown by fluorescence immunoassay (**Figure 3**), the expressions of HSP27 and 3 forms of phosphorylated HSP27 (hsp27-ser15, hsp27-ser78, hsp27-ser82) in the high glucose group and the control group decreased. The proteins were expressed in the cytoplasm and the nuclei, with high enrichment around the nuclei.

Expression of CaD and TM genes by QRT-PCR

The mRNA expressions of CaD and TM were 2.71±0.22E+07 and 1.10±0.09E+07 in the experimental group and 1.07±0.05E+07 and 0.51±0.02E+07 in the control group, respectively. As shown in **Table 2**, the differences in the expressions of CaD and TM between the experimental group and the control group reached a significant level ($P<0.05$); the expressions in the experimental group increased.

Expression of HSP27, phosphorylated HSP27, CaD and TM proteins by WB

SDS-PAGE gel images are shown in **Figure 4**. In the experimental group, the bands of HSP27 and phosphorylated HSP27 (hsp 27-ser15, hsp27-ser78 and hsp27-ser82) were weaker than those of the control group. However, the bands of CaD and TM were stronger than those of the control group. Using gel processing system, the grayscale value of each band was calculated along with the ratio to that of the internal reference. As compared with the control group, the expressions of CaD and TM proteins in the experimental group increased obviously, while those of HSP27 and phosphorylated HSP27 declined.

Discussion

DM is considered if any of the following criteria is met [10-13]: Fasting blood glucose above 7.0

Table 2. Expressions of genes in the experimental group and the control group

	CaD		TM		β-actin
	Average relative copy number (× 10^7)	Ratio	Average relative copy number (× 10^7)	Ratio	Average relative copy number (× 10^7)
Control group	1.07±0.05	0.13	0.51±0.02	0.06	8.32±0.85
Experimental group	2.71±0.22*	0.30	1.10±0.09*	0.12	8.99±1.16

Note: *P<0.05 compared with the control group.

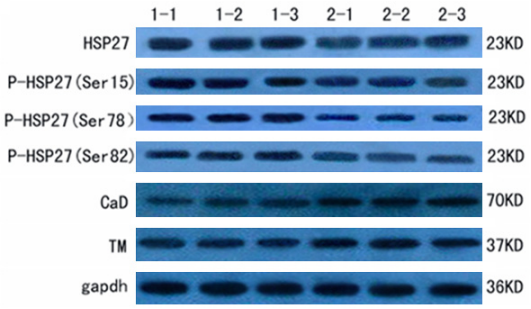

Figure 4. Expressions of HSP27, phosphorylated HSP27, CaD and TM by WB (1-1, 1-2 and 1-3 are the control group; 2-1,2-2 and 2-3 are the experimental group).

mM; glucose above 11 mM in 2-hour glucose tolerance test; HbAlc level above 48 mM; glucose at leisure time above 11.1 mM. According the criteria, we induced DM by adding 50 mM in rat DSMCs and set up the control group by adding 10 mM glucose. Then the expressions of HSP27, CaD and TM genes and proteins were detected.

HSP27 is an important chaperone and also the earliest discovered small-molecule heat shock protein [14]. According to the existing study [15], the upregulation of HSP27 expression and increased phosphorylation level in myometrium during late pregnancy and parturition are closely associated with enhanced uterine contractility. In airway smooth muscle cells, periodical mechanical traction can lead to increase of the HSP phosphorylation level, while maintain the stability of intracellular microfilaments and strengthen contractility of myocytes [16]. It can be inferred that the expression and activation of HSP27 in smooth muscle cells in response to stress are increased compensatorily and conducive to maintaining and restoring contractility of myocytes. HSP27 has 3 phosphorylation sites, which are Ser15, Ser78 and Ser82 [17].

CaD and TM are representative proteins related to thin myofilament contraction, which play an important role in regulating the contraction of smooth muscles. CaD has a length of 87 KD and binds to actin and TM. Since TM is located at the outer margin of actin monomer, it obstructs the myosin-binding sites on the actin sequence. As a result, the binding of the head of myosin to actin is blocked, thus regulating the contraction of smooth muscles [18, 19].

HSP27 can bind to the phosphorylated CaD, leading to the dissociation of CaD from TM and exposing the myosin-binding sites on the actin sequence. The binding of myosin to actin can regulate the contraction of intestinal smooth muscles [20]. In the rabbit DBD model, the upregulated expressions of CaD and TM and reduced contractility of DSMCs were observed [21]. In our study, the expressions of CaD and TM were increased under high glucose, which agreed with the above observation. The downregulated expressions of HSP27 and its phosphorylated forms may be due to the fact that the stress state was already over. However, the concrete regulatory mechanism is unknown. We built the DBD model in rat DSMCs in an attempt to understand the pathogenesis of DBD, to find measures to prevent DBD, to identify the target molecules and to evaluate prognosis.

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Disclosure of conflict of interest

None.

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