Increased proliferation of human bladder smooth muscle cells is mediated by physiological cyclic stretch via the PI3K-SGK1-Kv1.3 pathway

YE TIAN*, XUAN YUE*, DEYI LUO, ROMEL WAZIR, JIANZHONG WANG, TAO WU, LIN CHEN, BANGHUA LIAO and KUNJIE WANG

Department of Urology, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China

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Abstract. It is well known that specific mechanical stimuli induce positive changes in the physiological function and status of a number of cell types. However, an in-depth understanding of the application of mechanical forces has yet to be developed. The aim of the present study was to explore the optimal elongation and frequency of stretch-induced proliferation of human bladder smooth muscle cells (HBSMCs) and to investigate the mechanism involved in this process. HBSMCs were seeded in a silicone membrane and subjected to cyclic stretch of 2.5, 5, 10 and 15% equibiaxial elongation at frequencies of 0.05, 0.1, 0.2, 0.5 and 1 Hz, respectively. Bromodeoxyuridine (BrdU) assays were used to detect the proliferative activity of each group. To further determine the mechanism of the cell proliferation process triggered by physiological cyclic stretch, the expression of PI3K/SGK1/Akt/Kv1.3 was investigated at the transcriptional and translational levels by RT-PCR and western blot analysis, respectively. Optimal physiological stretch was established as 5% elongation at a frequency of 0.1 Hz, whereby HBSMCs revealed a marked increase in proliferative activity compared with the other groups, including the non-stretched group, which served as the control ($P<0.05$). The expression of PI3K/SGK1/Kv1.3; however, not Akt, were upregulated by cyclic stretch as compared with the control group. When separately treated with inhibitors of SGK1 and Kv1.3, increased stretch-induced proliferation was largely eliminated. These results markedly indicate that cyclic stretch induces the proliferation of HBSMCs and the PI3K-SGK1-Kv1.3 pathway is involved in this process, either fully or at least partially, rather than its related pathway, PI3K-Akt.

Correspondence to: Dr Kunjie Wang, Department of Urology, West China Hospital, Sichuan University, 37 Guoxue Alley, Chengdu, Sichuan 610041, P.R. China
E-mail: tianye_scu@163.com

*Contributed equally

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Introduction

Worldwide, patients with end-stage bladder disease are treated with cystoplasty using their own gastrointestinal segments; however, serious complications are attributable to the absorptive, mucus-secreting epithelial lining in the urinary tracts. Practical and functional tissue engineering of the bladder represents an ideal substitute and a number of studies have focused on this process in previous years (1,2). However, simple accumulation of the cells and matrix does not generate satisfactory results (3) and organic combinations of these cells and the scaffolds [extracellular matrix (ECM)] remain to be identified.

It is well documented that appropriate mechanical stimulation is critical for gene expression and cell proliferation, differentiation, migration, function optimization and production of structurally suitable ECM components (4-9). Human bladder smooth muscle cells (HBSMCs) are constantly subjected to mechanical stimuli, including hydrodynamic pressure and stretch, during filling and voiding cycles. Our previous studies indicated that HBSMC proliferation is stimulated by hydrodynamic pressure (10,11). However, to the best of our knowledge, stretch, which is considerably more important than hydrostatic pressure in physiological conditions, is far from well explored and the mechanisms by which HBSMCs perceive exterior mechanical stimulation remain poorly defined. It is well known that all cellular life recognizes and responds to stimuli from the extracellular environment. Environmental sensing at the cellular level relies on signal transduction involving the binding of extracellular signaling molecules and ligands to cell surface receptors that trigger events inside the cell (12). Elucidation of the interactions between physiological cyclic stretch and these signal transduction pathways may be beneficial or even fundamental to functional tissue engineering of the urinary bladder, and have important implications for the development of interventions for cell remodeling diseases, including incontinence, overactive bladder and bladder outlet obstruction (BOO) (13-15). The PI3K pathway is one of the most common physiological and pathological pathways, and is involved in a number of processes, including cell prolifera-

tion, metabolism, survival and tumorigenesis. AKT and SGK1 are related downstream effectors of the PI3K cascade, sharing similar downstream targets and 45-55% homology in their catalytic domains (16-18). For a specific type of cell, which of the 'two sisters' is responsible for the proliferation remains controversial (19-24). Cell proliferation requires an increase in the expression and function of potassium (K^+) channels. Blockade of K^+ channels inhibits the proliferation of a number of cell types (23,25,26). The Kv1.3 channel represents a novel target for vascular diseases due to its important relationship with the proliferation of vascular smooth muscle cells (27). However, the regulation of HBSMC proliferation by cyclic stretch and the mechanism of this process remains undefined.

Based on these previous studies (10), the aim of the present study was to explore the correlation between appropriate cyclic stretch and HBSMC proliferation, and furthermore, to identify changes in PI3K, SGK1, AKT and Kv1.3 expression and activity during regulated proliferation induced by cyclic stretch. The results indicate that HBSMC proliferative activity is upregulated by physiological cyclic stretch and when the stretch was applied by 5% elongation and 0.1 Hz, HBSMCs were demonstrated to exhibit maximum proliferative activity. Expression of PI3K, SGK1 and Kv1.3 was observed to be significantly increased. By contrast, AKT expression was unchanged. Increased proliferative activity was eliminated following blockade of SGK1 and Kv1.3. In addition, increased Kv1.3 expression was downregulated following blockade of SGK1, indicating that the PI3K-SGK1-Kv1.3 pathway is involved, at least in part, in HBSMC proliferation induced by cyclic stretch.

Materials and methods

Cell culture and identification. HBSMCs (ScienCell, Carlsbad, CA, USA; cat. no. 4310) were grown and maintained at 37˚C, in a 5% CO_2/95% air gas mixture and humidified atmosphere in a cell incubator. Dulbecco's modified Eagle's medium (DMEM; low glucose) was supplemented with 10% fetal bovine serum (both Hyclone Laboratories, Inc., Logan, UT, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). All experiments were performed on cells between passages 3 and 7 with normal morphology and good activity, which were confirmed by immunocytochemistry (data not shown).

Application of cyclic stretch. HBSMCs were seeded onto a silicone membrane for 24 h and then subjected to cyclic stretch. Stretch was applied based on the physiological bladder cycles using the Bose BioDynamic (Bose Corporation, Eden Prairie, MN, USA). In the first 3 h, elongation of stretch rose gradually from 0 to 2.5% and in the next 1 h, stretch rose from 2.5 to 5, 10 or 15%, or was maintained at 2.5%. Following a rapid decrease, this 4-h stretch cycle was repeated 4 times. Next, the silicone membrane was maintained in a relaxed state for 8 h (simulation of bladder cycles during night time). After applied elongations were divided into groups, each group was subjected to 0.05, 0.1, 0.2, 0.5 and 1 Hz with a sine wave stretch pattern by a 1:1 stretch/relaxation ratio (see Table I).

Proliferation studies. Bromodeoxyuridine (BrdU) incorporation (Roche Diagnostics GmbH, Mannheim, Germany) was employed as a direct parameter of DNA synthesis to quantify cell proliferation according to the manufacturer's instructions. Briefly, HBSMCs from each group were harvested and suspended at a concentration of $4x10^5$ cells/ml in DMEM. The cell suspension was transferred into a 96-well plate, with 200 μl in each well. BrdU labeling reagent (final concentration, 10 μM) was added and the cells were reincubated for 3 h. Following centrifugation, culture medium was removed and cells were fixed by FixDenat. Next, anti-BrdU antibody (1:100) was added to bind the BrdU incorporated in newly synthesized cellular DNA. Proliferation was quantified by measuring the absorbance value at a wavelength of 450 nm using an uQuant ELISA microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

RNA expression profile. Total RNA was extracted using TRIzol and cDNA was synthesized with SuperScript II (both Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions, at 37˚C for 15 min and 85˚C for 5 sec. PI3K, SGK1, AKT and Kv1.3 mRNA were quantified by real-time PCR using the GAPDH housekeeping gene as an internal control. Real-time PCR was performed using the SYBR Premix Ex Taq reagent (Takara Bio, Inc.) and the Bio-Rad iQ5 machine (Hercules, CA, USA). The PCR conditions were programmed as 94˚C for 3 min and 40 cycles of 94˚C for 5 sec, 54˚C for 30 sec and 72˚C for 20 sec . PCR product quality was monitored using post-PCR melt curve analysis. The following primer sequences were used: GAPDH forward, 5'-GCTTCGCTCTCTGCTCCT-3' and reverse, 5'-CGCCCAATACGACCAAAT-3'; PI3K forward, 5'-TGGCCTTAGCTCTTAGCCAAACAC-3' and reverse, 5'-ATTGGAACACGGCCTTTGACA-3'; SGK1 forward, 5'-CTATGCTGCTGAAATAGC-3' and reverse, 5'-GTCCGAAGTCAGTAAGG-3'; AKT forward, 5'-TCGGCAAGGTGATCCTGGTGAA-3' and reverse, 5'-AGGCGGTCGTGGGTCTGGAAAG-3'; Kv1.3 forward, 5'-AGTATATGGTGATCGAAGAGG-3' and reverse, 5'-AGTGAATATCTTCTTGATGTT-3'.

Western blot analysis. Expression of AKT/p-AKT, SGK1/p-SGK1 and Kv1.3 in HBSMCs was analyzed by western blot analysis, using GAPDH as an internal control. Briefly, total cells on the silicone membrane (with or without stretch) were harvested and then stored at -70˚C. Protein extracts were obtained from HBSMC samples treated with cell lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 μg/ml PMSF and 1% Triton X-100] for 30 min on ice. Following removal of cell debris by centrifugation (4˚C, 12,000 x g, 5 min), the lysate sample was boiled for 5 min in sample buffer, separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with specific primary antibodies at 4˚C overnight, followed by secondary anti-rabbit IgG (Jackson Immunoresearch Inc., West Grove, PA, USA) for 1 h. Reactive protein was detected by exposure on BioMax MR-1 film (Kodak, Rochester, NY, USA).

Statistical analysis. BrdU and RT-PCR assays were performed at least in triplicate, yielding similar results. Data are presented as the mean ± SD. Statistical significance was analyzed by one-way ANOVA test. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Absorbance values of BrdU incorporation.

Frequency, Hz	0% (control)	2.5%	5%	10%	15%
0.05	0.826±0.019[a]	0.965±0.035	1.264±0.029	1.281±0.053	1.235±0.026
0.10		1.161±0.033	1.460±0.015[b]	1.330±0.028	1.226±0.045
0.20		1.112±0.023	1.213±0.020	1.204±0.017	1.178±0.036
0.50		1.095±0.019	1.240±0.052	1.018±0.025	1.014±0.041
1.00		1.048±0.031	1.074±0.074	0.974±0.028	0.981±0.016

[a]P<0.05, vs. stretch; [b]P<0.05, vs. other groups. Optimal stretch model for HBSMC proliferation was established. Results of four independent BrdU incorporation assays in each group with various elongations and frequencies 30 min following the addition of anti-BrdU antibody. HBSMC, human bladder smooth muscle cell; BrdU, bromodeoxyuridine.

Results

Cyclic stretch increases proliferation. To investigate the proliferative activity of HBSMCs with or without stretch, the BrdU assay was performed. The correlation between absorbance values and proliferative activities was investigated in each group with a peak at 30 min following the addition of the anti-BrdU antibody. As demonstrated in Table I, proliferative activity was enhanced in each group compared with the control (elongation = 0). At a specific frequency (e.g., 0.1 Hz), proliferative activity was increased from 2.5 to 5% elongation; however, gradually decreased following 5%, indicating 5% elongation is the optimal magnitude of stretch for HBSMC proliferation (Fig. 1A). Similarly, when cyclic stretch was performed at a specific elongation (e.g., 5%), maximum proliferative activity was identified at 0.1 Hz, indicating that 0.1 Hz is an ideal parameter of cyclic stretch (Fig. 1B). Following the examination of proliferative activity induced by cyclic stretch, the simulated optimal physiological stretch (5% elongation, 0.1 Hz) was established. All subsequent stretches were performed based on these results.

Activation of the PI3K-SGK1-Kv1.3 pathway by cyclic stretch. To determine the possible mechanism of proliferative activity in response to the physiological stretch applied, the expression levels of proteins involved in the PI3K pathway, including AKT and SGK1 (the two main related downstream targets which regulate cell survival and proliferation) were assessed. Significant upregulation of PI3K (3.75±0.56-fold, P<0.05) and SGK1 (11.47±1.09-fold, P<0.05); however, not AKT (1.17±0.14-fold, P>0.05), was observed compared with the non-stretch group. In addition, mRNA expression of Kv1.3, which is responsible for proliferation, was increased by 3.05±0.30-fold (P<0.05; Fig. 2A). mRNA expression levels were verified with western blot analysis. In response to cyclic stretch, SGK1, p-SGK1 and Kv1.3 protein levels were significantly increased; however, AKT gene expression and activation did not respond to stretch stimulation (Fig. 2B). These results indicate that the PI3K-SGK1-Kv1.3 pathway, but not the PI3K-AKT pathway, is involved in stretch-induced proliferation of HBSMCs.

Inhibitors of SGK-1 and Kv1.3 eliminate the increase in proliferative activity. The role of the PI3K-SGK1-Kv1.3 pathway in stretch-induced HBSMC proliferation was further confirmed using SGK-1 and Kv1.3 inhibitors. Cyclic stretch was applied following exposure of the cells to the SKG1 inhibitor, CKI-7 dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA), at a final concentration of 100 μM. Proliferative activity was significantly suppressed compared with the stretch group without SGK1 inhibitor (0.986±0.042 vs. 1.460±0.015, P<0.05; Fig. 3A). In addition, Kv1.3 protein expression levels were analyzed by western blot analysis and no differences between

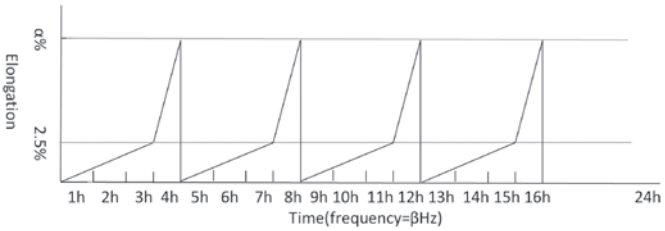

Figure 1. The application of cyclic stretch. Readouts of real-time representative elongation vs. time of stretch profile (α=2.5, 5, 10, 15%. β=0.05, 0.1, 0.2, 0.5 and 1Hz, respectively).

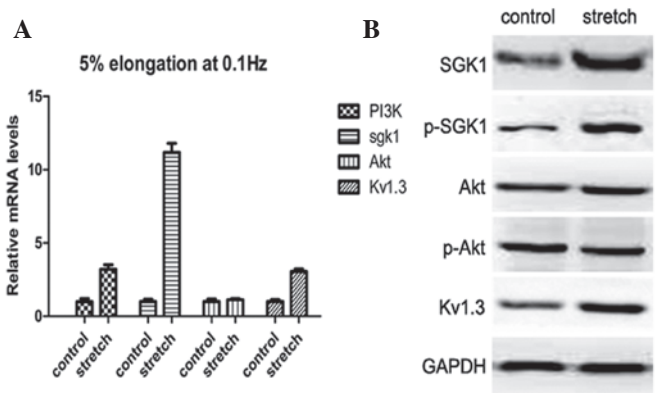

Figure 2. Expression of PI3K, SGK1, p-SGK1, AKT, p-AKT and Kv1.3 in the control and optimal stretch groups. (A) Real-time PCR of relative mRNA expression of PI3K, SGK1, AKT and Kv1.3 in HBSMCs with or without stretch. Expression of PI3K, SGK1 and Kv1.3; however, not AKT, was significantly increased compared with the corresponding controls. (B) Western blot analysis revealed the relative protein expression of SGK1, p-SGK1, AKT, p-AKT and Kv1.3. GAPDH was used as an internal control. Consistent with real-time PCR, expression of SGK1, p-SGK1 and Kv1.3; however, not AKT, was markedly elevated by stretch stimuli. HBSMCs, human bladder smooth muscle cells.

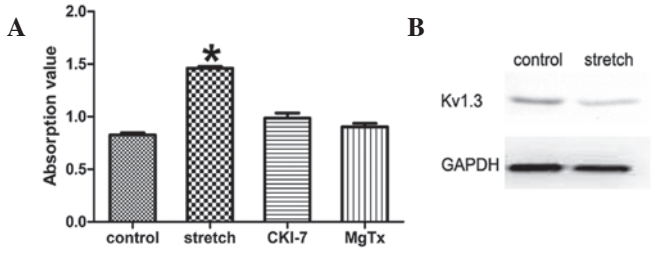

Figure 3. Absorbance values of BrdU incorporation assays following treatment with SGK1 and Kv1.3 inhibitors. (A) Stretch-induced proliferative activity was abolished by SGK1 and Kv1.3 inhibitors. *P<0.05, vs. inhibitor groups. Roles of SGK1 and Kv1.3 on stretch-induced HBSMC proliferation were confirmed. (B) Elevated Kv1.3 protein level was abolished by SGK1 inhibitor. No differences between control and stretch groups in Kv1.3 protein levels were found indicating that Kv1.3 is a downstream target of SGK1. HBSMC, human bladder smooth muscle cell; MgTx, margatoxin.

the stretch and non-stretch group was identified (Fig. 3B). Similarly, when the high-affinity blocker of Kv1.3, margatoxin (Sigma-Aldrich), was used at a final concentration of 10 nM, the increased proliferative activity was largely eradicated compared with the same stretch group; however, without the Kv1.3 inhibitor (0.902±0.030 vs. 1.460±0.015, P<0.05; Fig. 3A). These results indicate that Kv1.3 is a downstream target of SGK1 which, in turn, is responsible for HBSMC proliferation induced by physiological cyclic stretch.

Discussion

Due to the unique properties and functions of the human bladder compared with the gastrointestinal segments, the generation of functional bladders using tissue engineering is important for organ regeneration and replacement. However, there are a number of technical limitations associated with bladder tissue engineering (28). HBSMCs are fundamental for bladder tissue engineering, but HBSMCs are an inadequate source and show dysfunctional contractility in the tissue engineered urinary bladder without appropriate external stimuli. Mechanical stimuli, including stretch and hydrostatic and hydrodynamic pressure, are crucial for the maturity of these mechanically sensitive cells. Stretch is considerably more important than hydrostatic pressure and other mechanical stimuli for bladder tissue engineering (9).

Although previous studies have attempted to apply stretch to SMCs, the majority of these stretches were not designed based on physiological conditions (29). The proliferative effects varied with different strains in magnitude and frequency, thus making normative application of stretch imperative. With the two key parameters (elongation and frequency) defined, in the present study, optimal simulated physiological cyclic stretch for HBSMC proliferation was established. When cyclic stretch was applied at 5% elongation and 0.1 Hz, a significantly augmented proliferative activity was detected (1.460±0.015, P<0.05) compared with the other groups. To the best of our knowledge, this study is the first to report the optimal stretch parameters for HBSMC proliferation. Identification of the optimal proliferation stretch model is beneficial to the solution of cell source inadequacy and is also important for the elucidation of the pathophysiological mechanisms involved in diseases associated with HBSMC overproliferation.

A vast number of kinases in the cell constitute a complicated but specific network in which kinases activate their own pathways and interact with other pathways. Located downstream from PI3K, SGK1 and AKT are considered to be extremely similar kinase proteins (19). As proteins implicated as mechanotransduction mediators of HBSMC proliferation, the expression of PI3K, AKT, SGK1 and Kv1.3 was investigated at the transcriptional and translational levels. Results of RT-PCR revealed increased mRNA expression of PI3K, SGK1 and Kv1.3, but not AKT, consistent with the results of western blot analysis. Initially, we hypothesized that PI3K-SGK1-Kv1.3 was responsible for stretch-induced proliferation of HBSMCs. Inhibitors of SGK1 and Kv1.3 largely eliminated the proliferative activity and confirmed the role of this pathway. These results indicated that SGK1, but not AKT, is a mediator of mechanical signaling events in HBSMC proliferation. These observations are consistent with the results of a previous study in which SGK1 predisposed vasculature smooth muscle to an increased proliferative response to mechanical stimuli and this proliferative response was markedly suppressed by SGK1 knockout (24). In addition, in the present study, Kv1.3 was identified to be involved in the stretch-induced proliferation process downstream of SGK1. As a dominant intracellular secondary messenger, increased concentrations of cytoplasmic Ca^{2+} are involved in the regulation of cell proliferation (30). K^+ channels have been hypothesized to be important for the maintenance of cell membrane potential, which, in turn, is required for correct function of the Ca^{2+} release-activated Ca^{2+} channel (31). The latter channel, which is highly sensitive to membrane potential, mediates Ca^{2+} entry upon stimulation of cells, a prerequisite for triggering cell proliferation (32,33).

PI3K-AKT is a critical signaling pathway in the survival and proliferation of a number of cells (21,34) and is relatively well understood. However, SGK1, a 'sister' of AKT, has always been neglected. Functional analysis of gene-targeted mice lacking SGK1 provided insight into the functional significance of SGK1-dependent regulation of physiological functions. Knockout of SGK1 led to no severe phenotypes, indicating that SGK1 is not required for survival (35). Results indicated that SGK1 is a stimuli-responsive kinase that may mediate mechanical stretch-induced proliferation of HBSMCs, leading to bladder formation. Therefore, a hypothetical theory is raised: of the two 'sisters', AKT is implicated with basal proliferative activity and by contrast, SGK1 is responsible for the stimuli-induced activity. However, further confirmation and efforts must be performed to clearly illustrate this assumption.

In contrast to previous studies, several innovations were employed in this study: i) Cells were obtained from humans instead of animals; ii) physiological cyclic stretch was applied to stimulate the real bladder environment; iii) different elongations and frequencies were well studied, improved representative results were obtained and an optimized stretch model was established. Based on these results, we are likely to be able to provide qualified seed cells to bladder engineering techniques. In addition, observations indicate that the PI3K-SGK1-Kv1.3 pathway, but not PI3K-AKT, is the signal transduction pathway involved in stretch-induced proliferation and represents a promising pathway for novel targeted thera-

pies for specific urinary bladder diseases caused by excessive mechanical forces, including BOO. The use of drugs or inhibitors to inhibit SGK1 or Kv1.3 may provide promising methods to interrupt this pathological process. In particular, in the case of SGK1, which is not required for individual survival (35), 'block' treatment is likely be extremely effective, without severe complications.

Acknowledgements

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