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Expression and proliferation profiles of PKC, JNK and p38MAPK in physiologically stretched human bladder smooth muscle cells

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ABSTRACT

Objective: To determine protein kinase C (PKC), c-Jun NH2-Terminal Kinase (JNK) and P38 mitogen-activated protein kinases (p38MAPK) expression levels and effects of their respective inhibitors on proliferation of human bladder smooth muscle cells (HBSMCs) when physiologically stretched in vitro.

Materials and methods: HBSMCs were grown on silicone membrane and stretch was applied under varying conditions; (equibiaxial elongation: 2.5%, 5%, 10%, 15%, 20%, 25%), (frequency: 0.05, 0.1, 0.2, 0.5, 1 Hz). Optimal physiological stretch was established by assessing proliferation with 5-Bromo-2-deoxyuridine (BrdU) assay and flow cytometry. PKC, JNK and p38 expression levels were analyzed by Western blot. Specificity was maintained by employing specific inhibitors; (GF109203X for PKC, SP600125 for JNK and SB203580 for p38MAPK), in some experiments.

Results: Optimum proliferation was observed at 5% equibiaxial stretch (BrdU: 0.837 ± 0.026 (control) to 1.462 ± 0.023)%, ($P < 0.05$) and apoptotic cell death rate decreased from 16.4 ± 0.21% (control) to 4.5 ± 0.13% ($P < 0.05$) applied at 0.1 Hz. Expression of PKC was upregulated with slight increase in JNK and no change in p38MAPK after application of stretch. Inhibition had effects on proliferation (1.075 ± 0.024, $P < 0.05$ GF109203X); (1.418 ± 0.021, $P > 0.05$ SP600125) and (1.461 ± 0.01, $P > 0.05$ SB203580). These findings show that mechanical stretch can promote magnitude-dependent proliferative modulation through PKC and possibly JNK but not via p38MAPK in hBSMCs.

1. Introduction

Urine is stored at low intravesical pressures and expelled periodically. During these voiding cycles, the bladder wall is predisposed to range of mechanical stretches for which the BSMC must develop homeostatic mechanisms in order to regulate its biological activities [1]. These mechanisms are in turn co-dependent on a number of regulatory receptors and signaling pathways; including but not limited to: PI3K/AKt [2], p38 [3], STAT3 [4] and ERK1/2 [5].

Earlier we had shown the functional role of purinoceptors P2X1 and P2X2 in proliferation and contractility of the hBSMCs and how these receptors may be responsible for pathologic conditions with atropine resistance; like neurogenic and idiopathic detrusor over activity, bladder outlet obstruction, and interstitial cystitis/painful bladder syndrome [1].

PKC is another important regulatory protein in smooth muscle. PKC belongs to the sarcolemmal protein group and undergoes conformational changes caused by stretch, which may lead to activation of PKC itself [6]. Downstream signaling from activated PKC involves indirect or direct regulation of nuclear events [7]. Thus, PKC may act as a sensing molecule in response to mechanical stimulation.

Although conformational changes brought on by stretch in PKC have been investigated before, our study investigates these changes in normal BSMCs as compared with compensated/decompensated hypertrophied bladders and animal models. Secondly, we utilized three variables (elongation, frequency and time) encompassing all values previously reported in literature into a single hypothesis. Thirdly, BSMCs undergo a more static type of stretch as the bladder fills up [8]; and not sinusoidal or square pattern of stretch; observed in vascular SMCs. We have tried to replicate this static type of stretch to ensure more accurate resemblance of the physiologic stretching in bladder.

Similarly strain can activate Rac1 and its downstream MAPKs including MEK1/2, ERK1/2, JNK or p38MAPK [9–11]. Thus it is theoretically logical to hypothesize that JNK and p38MAPK may play a crucial role in cyclic stretch-induced proliferation of hBSMCs as well.

As we were already successful in replicating physiologic stretch model with optimum proliferation previously, we utilized this model again to study PKC, JNK, p38MAPK expression levels and

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see effects of their inhibition on the net proliferation. PKC had been shown previously as an important downstream effecter of cholinergic and purinergic signaling in stretch induced bladder cells (both pathways investigated previously in our lab) while JNK and p38MAPK are terminal effectors of Rac1 signaling pathway.

We will proceed from establishing physiologic stretch model, determining PKC, JNK and p38MAPK expression levels by western blot and finally assess the net proliferation under specific inhibitors.

2. Materials and methods

2.1. Materials

The HBSMCs were (ScienCell, USA, Cat. No. 4310). Cyclic strain was applied using BOSE Bioreactor (BOSE, BioDynamic, USA). The BrdU cell proliferation assay kit was purchased from Roche (Basel, Switzerland). anti-PKC, JNK and p38 antibodies were from Cell Signaling Technologies, (Boston, MA, USA).

2.1.1. Cell culture and cellular characterization

HBSMCs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95% air/5% CO_2. All experiments were performed on cells between passages 3 and 7.

2.1.2. Application of physiological stretch

In vitro physiologic stretch model was established previously [1]. Briefly; HBSMCs grown on a silicone membrane were subjected to stretch with the computer-controlled mechanical stretch unit (BOSE, BioDynamic, USA). bladder filling was recreated by applying elongation from 0% to 2.5% during first 3 h, followed by a progressive increase up to 5%, 10%, 15%, 20% or 25% during the last hour (completing a 4 h cycle) according to experiment designed. The bladder emptying was then mimicked by decreasing very quickly from 5%, 10%, 15%, 20% or 25% to 0%, in a few seconds. This stretch wave, articulated in three parts, being cyclically repeated 4 times in 24 h, whereas the cells remained at rest for the remaining time (8 h, in order to take into account the normal lower bladder activity during the night. HBSMCs in the control group were maintained in the same chamber without any stretch. Graphical representation of this stretch model is shown in Fig. 1. Real-time read-outs from the control software were recorded versus time.

2.1.3. Western blotting

Total cellular protein was extracted and pooled by using Nonidet P-40 (NP-40) protein isolation solution (0.5% NP-40, 60 mmol/L KCl, 1 mmol/L DTT, 10 mmol/L PMSF, 10 mmol/L Tris, pH 8.0, 1 mmol/L ethylene diaminetetraacetic acid, pH 8.0, and 1 mmol/L leupeptin, pepstatin and aprotinin). Protein concentration was calculated using ELISA Read. Membranes were probed with the following antibodies: GAPDH (as an internal control), PKC, JNK and p38 (all antibodies were 1:1000 diluted) After 2 h incubation with alkaline phosphatase conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) at room temperature, the signal was detected by the enhanced chemiluminescence method. Exposures were conducted on BioMax MR-1 film (Kodak, New York, NY, USA).

2.1.4. BrdU incorporation experiment for proliferation

After application of cyclic strain, cells were harvested and transferred at a density of 4.0×10^5 per well into 96-well plates. BrdU was added at a concentration of 10 μM to label the cells statically for another 3 h. After centrifugation (300*g* × 10 min), cells were fixed with FixDenat solution for 30 min at room temperature, and incubated with anti-BrdU peroxidase working solution (freshly diluted 1:100) for 90 min. Following 3 rinses with washing buffer, 100 μl/well substrate solution was added onto the cells and incubated at room temperature. Absorbance at 490 nm was measured at 5 min and every 10 min thereafter in an ELISA plate reader (Bio-Tek uQuant, USA). The optimal value was obtained at 30 min.

2.1.5. Flow cytometry analysis of cell cycle profile

Harvested cells were washed twice in cold PBS and fixed in 70% ethanol overnight at 4 °C. After centrifugation (25 °C, 1200 rpm × 3 min), cells were washed with cold PBS, and gently resuspended in 500 μl PBS containing 100 μg/ml RNaseA and 50 μg/ml propidium iodide for 30 min in dark. Cells were then diluted in PBS and flow cytometry was performed on a Cytomics FC500 (Beckman Coulter, CA, USA).

2.1.6. Statistical analysis

All experiments were performed in triplicates. Means ± SD were calculated. One-way ANOVA was applied to compare results between two groups. $P < 0.05$ was considered statistically significant. Figure data are expressed as the mean, the error bars indicates standard deviation.

3. Results

3.1. Expression of PKC, JNK and p38MAPK and effects of inhibition

Up-regulated expression was observed for PKC (2.490 ± 0.56-fold, $P < 0.05$), but JNK (1.004 ± 0.024-fold, $P > 0.05$) and p38MAPK (1.160 ± 0.004-fold, $P > 0.05$), protein levels were not significant compared with non-stretch group as shown in Fig. 2. PKC inhibitor failed to nullify the stretch effect (1.76 ± 0.112, $P > 0.05$), while JNK (SP600125, 0.833 ± 0.34) and p38MAPK (SB203580, 1.030 ± 0.14, $P < 0.05$) had no apparent effect. These data suggests a strong co relation between cyclic stretch and PKC while ruling out any role of JNK and p38MAPK in this process.

3.2. Stretch induces proliferation in magnitude and frequency dependent manner

In BrdU assay, the absorbance values directly correspond to amount of DNA synthesis and thus the proliferating cells. Proliferation activity was enhanced in each group compared with control (elongation = 0, 0.837 ± 0.026). The highest proliferation attained was 1.462 ± 0.023 at 5% stretch and 0.1 Hz frequency. 10%, 15%, 20%, 25% stretch group had considerably up-regulated proliferation (1.312 ± 0.035; 1.223 ± 0.028; 1.916 ± 0.065; 1.017 ± 1.019%; respectively) ($P < 0.05$), although proliferation values experienced a slow decline from 5% stretch group onwards to 25% stretch group; see Romel et al. [1].

Similarly, cyclic stretch inhibited apoptosis with S and G2/M phase cells increasing; being maximum at 5% stretch. Cells' apoptosis rate was calculated as: Proliferation index (%) = (S + G2/M)/(G0/G1 + S + G2/M) × 100%. The rate of apoptosis decreased from 16.4 ± 0.21% (control) to 4.5 ± 0.13% (stretch, $P < 0.05$), while cell proliferation index increased from 27.1 ± 0.74% (control) to 70.3 ± 0.22% (stretch, $P < 0.05$), as shown in Fig. 3; suggesting inhibition of apoptosis and stimulating proliferation of HBSMC.

All subsequent experiments were performed at this optimum stretch, i.e., 5% elongation and 0.1 Hz frequency.

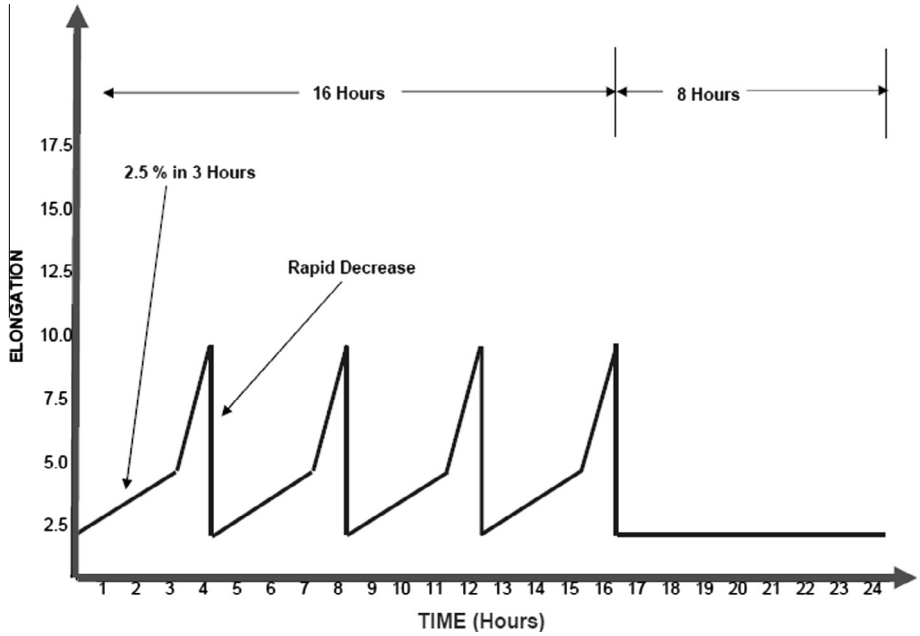

Fig. 1. Application of physiological stretch: elongation was applied from 0% to 2.5% during first 3 h, followed by a progressive increase up to 5%, 10%, 15%, 20% or 25% during the last hour (completing a 4 h cycle) according to experiment designed. The bladder emptying was then mimicked by decreasing very quickly from 5%, 10%, 15%, 20% or 25% to 0%, in a few seconds.

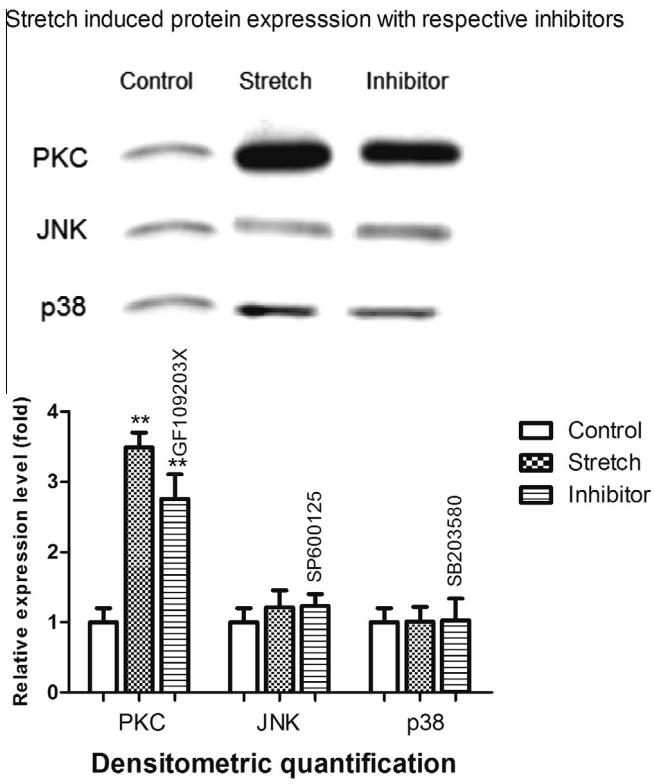

Fig. 2. Protein expression of PKC, JNK and p38: Western blotting results from a typical study with densitometric quantification. Stretch induces up regulation of PKC but no apparent change in JNK or p38. Shown values are the mean + SD from three independent experiments.

3.3. Role of PKC, JNK and p38MAPK in cyclic stretch induced proliferation

After establishing optimum physiologic stretch, the PKC, JNK and p38MAPK inhibitors: GF109203X, SP600125 and SB203580 respectively; were applied. Cells were incubated with 10 μM GF109203X, 20 μM SP600125 and 20 μM SB203580; 2 h before application of stretch in fresh medium and without addition of fetal bovine serum. Cells from stretch and control group were then assessed with BrdU assay (Fig. 4). Mean proliferation compared with control group (1.462 ± 0.023) decreased to (1.075 ± 0.024, $P < 0.05$ GF109203X); (1.418 ± 0.021, $P > 0.05$ SP600125) and (1.461 ± 0.01, $P > 0.05$ SB203580) as shown in Fig. 4. These findings show that mechanical stretch can promote magnitude-dependent proliferative modulation through PKC but not via JNK and p38MAPK in hBSMCs.

4. Discussion

In this experiment we used the well established stretch model; previously used to deduce a number of signally pathways and receptors in bladder smooth muscle cells. The usage of a large number of parameters which could affect the proliferation at cellular level were taken into account and systematically applied ranging from very low to as high as 25%.

To rule out apoptotic cell death, flow cytometry was performed and correlated well with the optimum stretch profile obtained at 5% equibiaxial stretch applied at 0.1 Hz.

This study concentrates on two diverse signaling families. JNK and p38MAPK are downstream regulators of Rac1, MEK1/2, ERK1/2 signaling pathway. While PKC is rendered catalytically competent by phosphorylations that correctly align residues for catalysis and localize it to the cytosol. Second, binding of ligands or, in some cases, substrate activates the enzyme by removing the pseudosubstrate from the substrate-binding site [12].

In bladder PKC, is subjected to modulation primarily by cholinergic but also purinergic and adrenergic signaling. To our knowledge it's the first study on PKC modulating cyclic stretch induced changes in bladder and interestingly, contrary to our results of human bladder JNK has been shown to modulate proliferation in animal models of bladder [6].

GF109203X is a potent and selective competitive inhibitor of PKC. It reduces the filling-state of intracellular Ins (1, 4, 5) P3 sensitive Ca2 + stores by inhibiting the Ca2 + uptake into these stores,

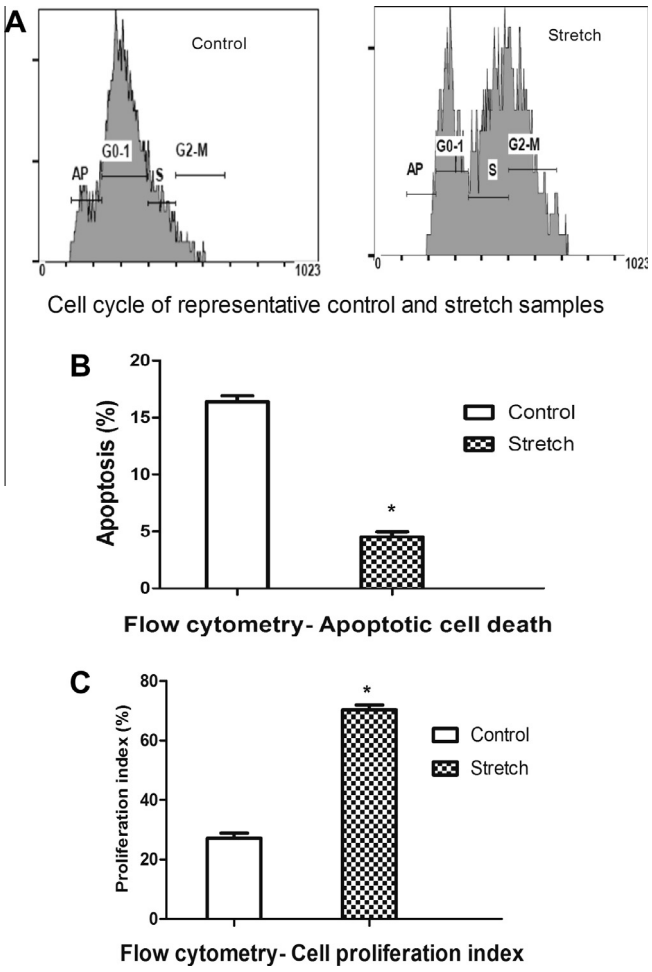

Fig. 3. Flow cytometry. (A) Shows cell cycle of control and stretched bladder smooth muscle cells. Application of stretch decreased the apoptotic cell death (B) while also increasing the cell proliferation index (C).

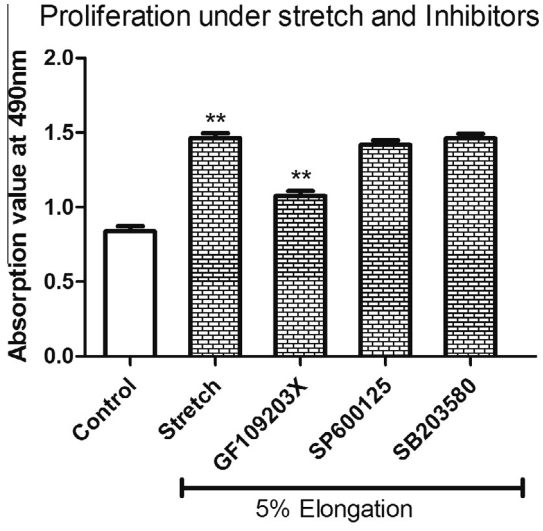

Fig. 4. Mean (+SD) proliferation of bladder smooth muscle cells. Figure shows absorbance values of 5-Bromo-2-deoxyuridine (BrdU) incorporation into the newly synthesized DNA and thus the proliferating cells. Maximum proliferation was observed at 5% elongation, while Gf109203X decreased proliferation, the JNK and p38 inhibitors, SP600125 and SB203580 had no effect. Implying a role for PKC but not JNK and p38MAPK.

thereby promoting store-dependent (capacitive) Ca2 + entry. Although inhibitory effects of GF109203X are not strong, it gives an alternative therapeutic option for modulating the quality and quantity of afferent information passed onto the central control areas from bladder. Similarly our study rules out the role of p38MAPK and more importantly JNK which is active in cellular modulation in animals but not in humans.

Additionally our stretch model can be exploited to further our knowledge of the complex signaling mechanisms and receptors involved in normal functioning of the human bladder and further our understanding of myriad pathologies such as interstitial cystitis, outflow obstruction and most types of neurogenic bladder.

5. Conflict of interest

No potential conflict of interest relevant to this article is reported.

Acknowledgments

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