The International Journal of Biochemistry & Cell Biology 59 (2015) 21–29

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Involvement of BK channel in differentiation of vascular smooth muscle cells induced by mechanical stretch

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ARTICLE INFO

Article history:
Received 7 August 2014
Received in revised form
13 November 2014
Accepted 25 November 2014
Available online 4 December 2014

Keywords:
Vascular smooth muscle cells
Differentiation
Mechanical stretch
BK channel
Endoplasmic reticulum stress

ABSTRACT

The differentiation of vascular smooth muscle cells (VSMCs), which are exposed to mechanical stretch *in vivo*, plays an important role in vascular remodeling during hypertension. Here, we demonstrated the mechanobiological roles of large conductance calcium and voltage-activated potassium (BK) channels in this process. In comparison with 5% stretch (physiological), 15% stretch (pathological) induced the de-differentiation of VSMCs, resulting in significantly decreased expressions of VSMC markers, *i.e.*, α-actin, calponin and SM22. The activity of BK channels, assessed by patch clamp recording, was significantly increased by 15% stretch and was accompanied by an increased alternative splicing of BK channel α-subunit at the stress axis-regulated exons (STREX). Furthermore, transfection of whole BK or STREX-deleted BK plasmids revealed that STREX was important for BK channels to sense mechanical stretch. Using thapsigargin (TG) which induces endoplasmic reticulum (ER) stress, and xbp1-targeted siRNA transfection which blocks ER stress, the results revealed that ER stress was contribute to stretch-induced alternative splicing of STREX. Our results suggested that during hypertension, pathological stretch may induce the ER stress in VSMCs, which affects the alternative splicing and activity of BK channels, and subsequently modulates VSMC differentiation.

1. Introduction

Vascular smooth muscle cells (VSMCs) in mature vessels are highly differentiated cells whose principal functions are contraction and regulation of blood vessel tone-diameter (Owens et al., 2004). There is strong evidence that the de-differentiation of VSMCs, defined by phenotypic transformation, abnormal proliferation, migration and synthetic capacity, contributes to pathogenesis of vascular remodeling (Parizek et al., 2011). It had been revealed that pathologically increased mechanical stretch, caused by the repetitive deformation of cells as arterial wall rhythmically distends and relaxes with blood pressure (Pfisterer et al., 2012), plays an important role in vascular remodeling during hypertension (Hoefer et al., 2013). However, the mechanism by which mechanical stretch modulates differentiation/de-differentiation of VSMCs remains to be elucidated.

Contractile proteins, such as α-actin, SM22α, calponin, and myosin heavy chain, have been shown to be specific differentiation markers and have the ability to maintain the contractility of VSMCs (Owens et al., 2004). Previous research found that 10% physiological stretch promotes VSMC differentiation through up-regulating of smooth muscle myogenesis (Qu et al., 2007), while pathological stretch induces VSMC de-differentiation with a concomitant down-regulation of contractile protein expression (Owens et al., 2004). It has been revealed that the increase of intracellular calcium initiates VSMC contraction, differentiation and proliferation (Kudryavtseva et al., 2013), which suggests that calcium may be a crucial molecule, and involved in the stretch-induced VSMC differentiation.

It has been proved that voltage gated calcium channels, including L-type and T-type, play important roles in VSMC differentiation *via* modulation of calcium influx (Kuhr et al., 2012; Kudryavtseva et al., 2013). The down-regulation of L-type calcium channels is accompanied with VSMC de-differentiation (Gollasch et al., 1998). T-type channels inactivation are faster than L-type channels, and Kuhr et al. (2012) proved that calcium influx through T-type

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http://dx.doi.org/10.1016/j.biocel.2014.11.011
1357-2725/

channels is associated with VSMC proliferation and differentiation. Beside the calcium influx *via* calcium channel, the calcium homeostasis is also regulated by calcium releasing from endoplasmic reticulum (ER), the crucial intracellular calcium store (Galva et al., 2012). Further researches revealed that calcium influx *via* calcium channels and calcium releasing from ER are both regulated by complex mechanism, including large conductance calcium and voltage-activated potassium (BK) channels (Joseph et al., 2013).

BK channels are predominantly expressed in VSMCs and play important roles in modulating vascular tone to regulate blood flow (Pang and Rusch, 2009). BK channel is composed of the α-subunit that forms the pore structure, and the accessory β-subunit (Sausbier et al., 2005). Increased expression of the BK channel α-subunit inhibits proliferation of endothelial cells under shear stress application, which suggests that BK channel may be mechanosensitive (Jia et al., 2013). It has been reported that BK channels may also participate in the modulation of VSMC differentiation (Long et al., 2009). However, the roles and mechanisms of BK channels in pathological stretch-induced VSMC de-differentiation are still unclear.

It has been shown that alternative splicing at the C terminal of the BK channel α-subunit is a major determinant of BK channel function (Ma et al., 2007). Naruse et al. (2009) revealed that stress axis-regulated exons (STREX) located at the C terminal of the BK channel α-subunit are necessary for chick myocyte mechanosensitivity. Therefore, we hypothesized that the alternative splicing of STREX in BK channel may contribute to the pathological stretch-induced de-differentiation of VSMCs during hypertension.

In the present study, to demonstrate the roles of the activity and mechano-sensitive alternative splicing of BK channel in VSMC differentiation modulated by mechanical stretch, VSMCs were subjected to 5% and 15% stretch *in vitro* to mimic physiological and pathological mechanical situations, respectively (Qi et al., 2010). The effects of different stretches on the expression and activity of BK channel, as well as on the differentiation and intracellular calcium levels in VSMCs, were also examined. Furthermore, the underlying mechanism of alternative splicing in response to the pathological stretch was studied. Studying the effects of BK channel on VSMC differentiation modulated by mechanical stimuli will help to understand the pathogenesis of hypertension.

2. Materials and methods

2.1. Cell culture

SD rats were euthanized with sodium pentobarbital at 120 mg/kg, and then thoracic aorta was surgically removed. Primary VSMCs were cultured from the medial portion of thoracic aortas after the removal of adventitia and endothelium as described in previous study (Qi et al., 2010). VSMCs from at least four isolations and in passages 3–8 were used for experiments.

Since there is no BK channel expression on natural HEK cells, HEK transfected with whole BK channel plasmids (HEK-Slo) or STREX-deleted BK channel plasmids (HEK-STREX delete) were used to explore the role of STREX splicing on BK activity (Ma et al., 2010). HEK cells were purchased from the Beijing Cancer Hospital and were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). Cells were grown in 95% air and 5% CO_2 at 37 °C.

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the protocol was approved by the Animal Research Committee of Shanghai Jiao Tong University.

2.2. Mechanical stretch application

For western blot and RT-PCR, FX-4000T Stretch Unit (Flexercell International, USA) was used to apply cyclic stretch. Briefly, VSMCs were seeded on collagen I-coated flexible silicone bottom plates (Flexercell International, USA) at a density of 3×10^5 cells per well (9.32 cm^2). The cells were serum starved for 24 h prior to cyclic stretch application. The following mechanical parameters were used: stretch magnitudes of 5% and 15% respectively, at a constant loading duration of 24 h and a frequency of 1.25 Hz.

For patch-clamp recording and calcium imaging, cells were subjected to mechanical stretch by horizontal stretch device as previously described (Ahmed et al., 2010). Briefly, 1 ml SYLGARD 184 (Dow Corning Corporation, USA) was poured onto the surface of a cover slip for 24–48 h at room temperature to form silicone gels with smooth surfaces, and these gels were then cut into slices for cell culture (Armbruster et al., 2009). VSMCs and HEK cells were trypsinized and seeded at a density of 3×10^5 cells per silicone gel slice, which was positioned onto flexible baseplates. Cells were serum starved for 24 h and then elongated by 5% or 15% as adjusted by the horizontal movement distance of the baseplates.

2.3. Plasmid transfection

To study the role of BK channel in VSMC differentiation, the GFP and whole BK channel plasmids were co-transfected into VSMCs using X-TREME GENE HP DNA Transfection Reagent (Roche). Briefly, after the cells reached 70% confluence in 24-well plates, VSMCs were incubated with a transfection mixture including 1 μg GFP plasmid, 4 μg BK channel plasmid, 4 μl Transfection Reagent (Roche), and 100 μl Opti-MEM (Invitrogen). The cultures were brought to a final volume of 500 μl without serum and antibiotics. After transfection for 24 h, the cells were used for experiments.

To study the role of STREX in BK channel subjected to mechanical stretch, GFP plasmid with whole BK channel or STREX-deleted plasmids were co-transfected into HEK cells (HEK-Slo or HEK-STREX delete) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 4 μg BK channel plasmid and 1 μg GFP plasmid or 4 μg STREX-deleted plasmid and 1 μg GFP plasmid were mixed with 5 μl Lipofectamine 2000 (Invitrogen) diluted in 100 μl Opti-MEM (Invitrogen) without serum and antibiotics. The mixture was added onto the cells in 6-well plates to a final culture volume of 800 μl. After transfection for 24 h, the cells were trypsinized, seeded on silicone gels and allowed to attach for 24 h prior to mechanical stretch.

2.4. RNA interference

VSMCs were seeded at a density of 2×10^5 cells per well on Flexercell plates and grown in 10% FBS/DMEM. After 24 h, the cells were transfected with siRNA targeting xbp1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Briefly, 100 nmol siRNA and 5 μl Lipofectamine 2000 (Invitrogen) were diluted in Opti-MEM (Invitrogen) without serum and antibiotics. After mixing for 20 min at room temperature, the mixture was added dropwise to a final volume of 800 μl, and the cells were incubated at 37 °C in a humidified incubator. After 24 h incubation, the interference mixture was removed, and complete culture medium was added. Non-silencing siRNA was used as a mock control. The sequences of the double stranded siRNA targeting xbp1 were 5′-GCUG UUGC CUCU UCAG AUUT T-3′ and 5′-AATC TGAA GAGG CAAC AGCT T-3′; the sequences for the mock control were 5′-UUCU CCGA ACGU GUCA CGUT T-3′ and 5′-ACGU GACA CGUU CGGA GAAT T-3′.