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Stimulation of vascular smooth muscle cell proliferation by stiff matrix via the IK_{Ca} channel-dependent Ca^{2+} signaling

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Running title: IK_{Ca} channel in matrix stiffness regulation of VSMC proliferation

ABSTRACT

Vascular stiffening, an early and common characteristic of cardiovascular diseases (CVDs), stimulates vascular smooth muscle cell (VSMC) proliferation which reciprocally accelerates the progression of CVDs. However, the mechanisms by which extracellular matrix stiffness accompanying vascular stiffening regulates VSMC proliferation remain largely unknown. In the present study, we examined the role of the intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}) channel in the matrix stiffness regulation of VSMC proliferation by growing A7r5 cells on soft and stiff polydimethylsiloxane substrates with stiffness close to these of arteries under physiological and pathological conditions, respectively. Stiff substrates stimulated cell proliferation and up-regulated the expression of the IK_{Ca} channel. Stiff substrate-induced cell proliferation was suppressed by pharmacological inhibition using TRAM34, an IK_{Ca} channel blocker, or genetic depletion of the IK_{Ca} channel. In addition, stiff substrate-induced cell proliferation was also suppressed by reducing extracellular Ca^{2+} concentration using EGTA or intracellular Ca^{2+} concentration using BAPTA-AM. Moreover, stiff substrate induced activation of extracellular signal-regulated kinases (ERK), which was inhibited by treatment with TRAM34 or BAPTA-AM. Stiff substrate-induced cell proliferation was suppressed by treatment with PD98059, an ERK inhibitor. Taken together, these results show that substrates with pathologically relevant stiffness upregulate the IK_{Ca} channel expression to enhance intracellular Ca^{2+} signalling and subsequent activation of the ERK signal pathway to drive cell proliferation. These findings provide a novel mechanism by which vascular stiffening regulates VSMC function.

KEY WORDS: Matrix stiffness; vascular smooth muscle cell proliferation; intermediate-conductance Ca^{2+}-activated K^+ channel; Ca^{2+}; extracellular signal-regulated kinases

INTRODUCTION

The blood vessel wall consists of multiple cell types, including vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM), and ECM is an important factor determining the mechanical properties or stiffness of blood vessels wall (Ebrahimi, 2009; Kohn, Lampi, & Reinhart-King, 2015; Matsumoto & Nagayama, 2012; Wagenseil & Mecham, 2009). Vascular stiffening is an early and common characteristic of cardiovascular diseases (CVDs), which are associated with high morbidity and mortality, such as hypertension, atherosclerosis and aneurism, and is positively correlated with the progession of CVDs (Cavalcante, Lima, Redheuil, & Al-Mallah, 2011; Ebrahimi, 2009; Hoffman et al., 2017; Laurent, Kingwell, Bank, Weber, & Struijker-Boudier, 2002; Llaurado et al., 2012; O'Rourke, Staessen, Vlachopoulos, Duprez, & Plante, 2002; Wykretowicz et al., 2009). VSMCs mainly constitute the middle layer of blood vessel wall, and play a pivotal role in maintaining the vasculature structure and function. The functions of VSMCs are known to be significantly regulated by environmental cues of blood vessels, and reciprocally contribute to the pathophysiological state of blood vessels. VSMCs in normal arteries exhibit a contractile phenotype and regulate vascular tone. However, in response to vascular stiffening, VMSCs can switch to a synthetic phenotype and excessively proliferate (Brown et al., 2010; Haga, Li, & Chien, 2007; House, Potier, Bisaillon, Singer, & Trebak, 2008; Sazonova et al., 2015). Such remarkable changes in cellular function were largely recapitulated in VSMCs when they were cultured on cell-supporting substrate matrix, with VSMCs exhibiting increased proliferation and the contractile-to-synthetic phenotype switch on stiff substrates (Brown et al., 2010; Peyton, Raub, Keschrumrus, & Putnam, 2006; Xie et al., 2018). However, the detailed mechanisms underlying such mechanical regulation of VSMC functions remain largely

elusive.

It is known that intracellular Ca^{2+} is a ubiquitous second messenger with a critical role in regulating diverse cell functions including proliferation (Ghosh et al., 2017; Hara et al., 2012; House et al., 2008; Shukla, Rowe, Hinton, Angelini, & Jeremy, 2005). It has been well documented that the contractile-to-synthetic phenotype switch of VSMCs is accompanied by salient changes in the expression profile of ion channels that are important in the modulation of intracellular Ca^{2+} signaling. Thus, in contractile VSMCs, the big-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel is highly expressed, but its expression strongly declines or even disappears in synthetic VSMCs, in which the expression of the intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}, also known as $K_{Ca3.1}$ or SK4) channel is substantially upregulated (Ghosh et al., 2017; Gonzalez-Cobos & Trebak, 2010; Guo et al., 2017; House et al., 2008; Kumar et al., 2006; Si et al., 2006; Tharp, Wamhoff, Turk, & Bowles, 2006). Previous studies showed that pharmacological blockade or genetic depletion of the IK_{Ca} channel effectively suppressed VSMC proliferation induced by platelet-derived growth factor and alleviated the development of atherosclerosis and restenosis (Bi et al., 2013; D. L. Tharp, 2006; Si et al., 2006; Tharp et al., 2006; Tharp et al., 2008; Toyama et al., 2008). In human erythroleukemia cells, prostate cancer cells, chondrosarcoma cells and mesenchymal stem cells (MSCs), it has been shown that activation of the IK_{Ca} channel can increase the driving force for Ca^{2+} entry to raise intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) via membrane hyperpolarization (Fioretti et al., 2009; Funabashi et al., 2010; Lallet-Daher et al., 2009; Tarasov et al., 2017; Xinghua Lu, 1999). In addition, there is evidence to suggest that IK_{Ca} channel-dependent Ca^{2+} signaling regulates cell proliferation in prostate cancer cells (Lallet-Daher et al., 2009). While similar molecular mechanisms have been hypothesized for the

IK_{Ca} channel in VSMCs in the regulation of cell proliferation (House et al., 2008), the supporting evidence is limited. Furthermore, a recent study shows the importance of Ca^{2+}-dependent downstream signaling pathways, such as mitogen-activated protein kinases (MAPKs), particularly extracellular signal-regulated kinase (ERK) in the regulation of cell proliferation in epithelial cells (Gudipaty et al., 2017). It remains unclear whether such a Ca^{2+}-dependent downstream signalling pathway is engaged in mechanical regulation of VSMC proliferation.

We and other groups have shown that the expression and activity of the IK_{Ca} channel in VSMCs and other cell types such as endothelial cells and MSCs are sensitive to regulation by mechanical forces, such as shear stress or membrane stretch (Brakemeier et al., 2003; Hayabuchi et al., 2011; Takai et al., 2013; Jia et al., 2020). It is interesting to examine whether vascular stiffening-related mechanical stimulation regulates the IK_{Ca} channel expression and activity in VSMCs and thereby VSMC proliferation. Therefore, in this study we investigated the effects of substrate stiffness on the IK_{Ca} channel and cell proliferation by growing rat aorta A7r5VSMCs, on polydimethylsiloxane (PDMS) substrates with stiffness similar to that of blood vessels under physiological and pathological conditions. Our results show stiff substrates strongly upregulate the IK_{Ca} channel expression and activity that are critical in substrate stiffness-dependent cell proliferation. Furthermore, we provide evidence to suggest that IK_{Ca}-mediated increase in the intracellular Ca^{2+} concentration and activation of Ca^{2+}-dependent ERK signal pathway are important in mediating substrate stiffness regulation of VSMC proliferation. These results provide evidence to show a novel molecular mechanism that may contribute to vascular stiffening-induced VSMC proliferation and related pathogenesis and progression of CVDs.

MATERIALS AND METHODS

Preparation and characterization of PDMS substrates

PDMS substrates with varying stiffness were fabricated through blending sylgard 184 gel and sylgard 527 gel (Dow Corning, USA) as previously described (Palchesko, Zhang, Sun, & Feinberg, 2012). Briefly, base liquid and curing agent of sylgard 184 gel were mixed at a mass ratio of 10:1, while the part A and B of sylgard 527 gel were mixed equally. Subsequently, these two gels were blended with varying mass percentage of sylgard 184 from 0 to 100. Once defoamed in a Thinky-Conditioning mixer (Thinky Corporation, Japan), PDMS was poured into tissue culture plates to create ~2-mm thick films. All substrates were cured at 100^{o}C for 4 h, followed by treatment with UV-Ozone cleaner (Novascan Technologies, Ames, IA, USA) for 30 min and immediately coating with 25 μg/ml fibronectin (FN) (Corning) for 2 h before use for cell culture. FN coating was confirmed by fluorescence microscopic imaging of Alexa Fluor® 647 mouse anti-FN (BD Biosciences, San Jose, CA).

The Young's modulus of PDMS substrates was determined by uniaxial tensile testing using a Shimadzu universal tester (Shimadzu, Japan) as previously described (Zhang et al., 2014). Substrate topography was examined using an atomic force microscope (AFM) of Scanning Near-field Optical Microscopy (SNOM, NTEGRA Solaris, NT-MDT, Russia) in tapping mode with a scan point size of 256 × 256 over an area of 5 μm × 5 μm.

Cell culture and treatment

A7r5 cells, a rat aorta VSMC line widely used as a cell model of VSMCs, were obtained from ATCC

(American Type Culture Collection) and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Cells were seeded on PDMS substrates. In some cases, cells were cultured in medium containing 100 nM TRAM34 (Santa Cruz, CA, USA) to block the IK_{Ca} channel, 1 mM EGTA to reduce extracellular Ca^{2+} concentration, 10 μM BAPTA-AM (AbMole Bioscience) to chelate intracellular Ca^{2+} concentration, or 20 μM PD98059 (Cell Signaling Technology, USA) to inhibit ERK, respectively.

F-actin staining and microscopy

Cells were seeded on circular glass coverslips that were covered with defined PDMS substrates and placed in a 6-well plate at a density of 3×10^4 cells/well, and incubated for 24 h. After fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton-X, cells were incubated with rhodamine-labeled phalloidin (1:40, Invitrogen) for 20 min. DAPI (1:1000) was used to stain nuclei. Images were captured using a confocal laser scanning microscope (Leica, Germany). Cell spreading area was calculated by Image J.

Cell proliferation

Cells proliferation was quantified by Cell Counting Kit-8 (CCK-8, Beyotime, China) assay. Cells were seeded on 24-well plates covered with defined PDMS substrates at a density of 3×10^4 cells/well. After incubation for 1, 2, 4 or 6 days, CCK-8 solution was added to culture media with 1:10 ratio and

incubated for 4 h in an incubator. After that, 100-μl solutions per well were added into 96-well plates, and the optical density value at 450 nm (OD_{450}) was measured using a plate reader (Thermo Fisher, USA).

Cell cycle analysis

Cells were seeded on 6-well plates covered with defined PDMS substrates at a density of 8×10^4 cells/well. Cells, after synchronized with serum-free medium for 24 h, were cultured for another 24 h in the presence of 10% FBS, and collected and fixed in 70% ethanol. Following three washes with phosphate buffer saline (PBS), cells were incubated in PBS containing 20 μg/ml propidium iodide (PI) and 100 μg/ml RNase A at 37°C for 30 min. Cell cycle distributions were determined by FACSCalibur (Becton Dickinson) and analyzed using the ModFit software.

RT-PCR

Cells were seeded on 24-well plates covered with defined PDMS substrates at a density of 3×10^4 cells/well and cultured for 2 days. Total RNA was extracted using a Trizol RNA-prep kit (Qiagen) according to the manufacturer's instructions. The RNA concentration and purity were determined using a Nanodrop2000 spectrophotometer (Thermo Scientific). One μg RNA was reverse-transcribed into cDNA in a 25-μl reaction volume using a High Capacity RNA-to-cDNA kit (Applied Biosystems) and a Mastercycler Gradient PCR machine (Thermo scientific) at 37ºC for 60 min. The reverse transcription was stopped at 95ºC for 5 min. One μl cDNA was used for PCR in a 10-μl reaction volume using a PCR kit (YTHX Biotechnology, China). The primers (Invitrogen) used are:

5’-CTCCTTTGTCTTATTGTGGTCTTCC-3’ (forward) and 5’- GGTGGTCCAGGGTTTCTTA -3’ (reverse) for IK_{Ca}, and 5’- GAACAAGTGAATTCCATGGT-3’ (forward) and 5’-CTATGTGGCCTCCTGGATG-3’ (reverse) for GAPDH. PCR was performed using the following conditions: 94°C for 5 min, 35-40 cycles (94°C for 30 s; 56°C for 30 s; 72°C for 20 s), and 72°C for 5 min. PCR products were separated on 2% agarose gels containing ethidium bromide and visualized with a UV transilluminator (Tanon, China). GAPDH was used as an internal control. The intensity of the PCR products was analyzed by BandScan.

Determination of cell surface protein expression

The IK_{Ca} protein expression on cell surface was determined by flow cytometry as previously described (Jia et al., 2013). Briefly, cells were seeded on 6-well plates covered with defined PDMS substrates at a density of 8×10^4 cells/well and cultured for 2 days. Cells were harvested and fixed with 4% paraformaldehyde for 30 min. Following three washes with PBS, cells were incubated with a primary mouse antibody specifically against the IK_{Ca} extracellular domain (ALM051, 1:100, Alomone Labs, Israel) for 40 min. Cells were incubated with the irrelevant mouse isotope antibody (1:100, Alomone Labs, Israel) instead of specific antibody as negative control (NC). After three washes with PBS, cells were incubated with a secondary fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (1:200, Santa Cruz Biotechnology) for 30 min in dark. The fluorescence intensity was detected by flow cytometer (FACSCalibur, BD) on FL-1 channel and the fluorescence intensity was quantified by CellQuest software. The IK_{Ca} expression was derived by subtracting the fluorescence intensity of NC (as shown by the dotted line in Fig. 2B) from the fluorescence intensity of specific-binding.

Transfection with siRNA

IK_{Ca}-specific siRNA ($siIK_{Ca}$) (sense: 5'-GGAGGUCCAGCUGUUCAUGtt-3', antisense: 5'-CAUGAACAGCUGGACCUCCtt-3') and a scrambled control siRNA (ctrl siCTL) (sense: 5'-CAUUCACUCAGGUCAUCAGtt-3', antisense: 5'-CUGAUGACCUGAGUGAAUGtt-3') were synthesized by GenePharma (Shanghai, China). Cells were seeded on 6-well plates at a density of 8×10^4 cells/well and transfected with 25 nM of $siIK_{Ca}$ or siCTL using siPORTt Amine (Ambion) according to the manufacturer's protocols. Cells 24 hr post transfection were seeded on PDMS substrates for further experiments.

Western blotting

Cells were seeded on 6-well plates covered with defined PDMS substrates at a density of 8×10^4 cells/well and incubated for 1 day. Total proteins were harvested by radio-immunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) supplemented with 2% (v/v) phenylmethanesulfonyl fluoride (Sigma), and quantified by a bicinchoninic acid kit (Beyotime, China). Sixty μg proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Sigma). After blocked using 5% non-fat milk for 2 h at room temperature, membranes were incubated with primary rabbit antibodies against-IK_{Ca} (1:100), p-ERK (1:1000), ERK (1:1000), or GAPDH (1:5000) (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by incubation with a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5000, from Zhongshan Biotechnology, China).

Proteins were visualized using enhanced chemiluminescence (Applygen, Beijing, China) and images were captured by MiniChemi (Sagecreation, China). GAPDH was used as load control. The intensity of proteins was analyzed by Image J.

Determination of $[Ca^{2+}]_i$

Single cell Ca^{2+} imaging and the Ca^{2+} fluorescence indictor Fluo4 were was used to monitor the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ as previously described (Jia et al., 2013). Briefly, after cultured on defined PDMS substrates for 24 h, cells were loaded with 5 μM Fluo4-AM (Molecular Probes, USA) for 40 min at room temperature in extracellular Ca^{2+}-containing solutions containing (in mM): 140 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.13 $MgCl_2$, 10 glucose and 10 HEPES. After washes with extracellular solutions, cells in ~30 view fields were imaged using a confocal microscope (Leica, Germany). The Fluo4 fluorescence intensity in each cell was analyzed using Image J. The relative Fluo4 fluorescence intensity in individual cells, defined by $(F-F_0)/F_0$, was used to indicate the $[Ca^{2+}]_i$ in individual cells (Figure 4B); F is the fluorescence observed from individual cells and F_0 is the background fluorescence. In some experiments, cells were treated with 100 nM TRAM34.

Statistical analysis

Data are presented as mean ± standard derivation, where appropriate. Student's t-test was used for comparison between two groups, and one-way ANOVA followed by *post hoc* Fisher's test for comparison of multiple groups. A *P*-value of < 0.05 was considered significant.

RESULTS

Stiff substrates stimulate VSMC proliferation

To investigate whether matrix stiffness regulates VSMCs, we prepared PDMS substrates with Young's modulus close to that of the vasculature reported under physiological conditions (0.21 MPa) or under pathological conditions of vascular stiffening (0.77 MPa and 1.72 MPa) (Fig. 1A) (Ebrahimi, 2009; Hoffman et al., 2017; Laurent, et al., 2002) and cultured A7r5 cells on such substrates. AFM imaging revealed a similar topography for these substrates with different stiffness (Fig. 1B). Cells growing on the substrates of 0.77 MPa and 1.72 MPa exhibited noticeably greater spreading, as compared to those on the substrate of 0.21 MPa (Fig. 1C), providing an initial indication that cells sensed and responded to different substrate stiffness. As introduced above, vascular stiffening is associated with the highly proliferative phenotype of VSMCs. We therefore analyzed cell proliferation after cultured on these three substrates for 1, 2, 4 and 6 days. There was no significant difference in cell proliferation on the two stiff substrates, but cells on these substrates proliferated significantly faster than those on the soft substrate, which was consistently observed at all time points examined (Fig. 1D). Furthermore, flow cytometric analysis revealed that the cell cycle distribution remained similar for cells on the two stiff substrates, with increased percentage of cells in the S phase and reduced percentage of cells in the G0/G1 phase, as compared to those for cells on the soft substrate (supplemental data Fig. S1), suggesting that stiff substrates stimulate the cell cycle progression.

Stiff substrates upregulate the IK_{Ca} channel expression and activity to increase cell proliferation

It is known that VSMC proliferation and phenotype switch are accompanied with upregulation of the IK_{Ca} expression (Bi et al., 2013; Si et al., 2006; Tharp et al., 2006). We next examined whether the IK_{Ca} channel expression was regulated by substrate stiffness. Semi-quantitative RT-PCR showed that the IK_{Ca} mRNA expression level in cells on the two stiff substrates was substantially higher than that in cells on the soft substrate (Fig. 2A and C). Consistently, analysis using flow cytometry in combination of an antibody recognizing the extracellular part of the IK_{Ca} protein also indicates the cell surface IK_{Ca} protein expression level in cells on the two stiff substrates was significantly higher than that in cells on the soft substrate (Fig. 2B and C). There was slight but statistically insignificant difference in the IK_{Ca} mRNA and protein expression levels in cells on the two stiff substrates (Fig. 2A-C), similar to the effects of substrate stiffness on cell proliferation described above. Therefore, further experiments were carried out using the substrates with 0.21 MPa and 1.72 MPa, which from here onwards are referred to as the soft and stiff substrates, respectively.

It has been reported that the IK_{Ca} channel is positively associated with VSMC proliferation. Therefore, we were interested in whether such substrate stiffness upregulation of the IK_{Ca} channel was critical for substrate stiff-dependent increase in cell proliferation. Treatment with TRAM34 was without effect on cell proliferation on the soft substrate but almost completely prevented the increase in cell proliferation on the stiff substrate (Fig. 3A). Similarly, treatment with IK_{Ca}-specific siRNA to reduce the IK_{Ca} expression (Fig. 3B) strongly suppressed such substrate stiffness-dependent increase in cell proliferation (Fig. 3C). Taken together, these pharmacological and genetic studies provide consistent evidence to support that upregulation of the IK_{Ca} channel is an important mechanism for stiff substrate-induced increase in VSMC proliferation.

IK_{Ca}-dependent increase in $[Ca^{2+}]_i$ is required for stiff substrate-induced increase in VSMC proliferation

It is known that the IK_{Ca} channel, while its activation is sensitive to intracellular Ca^{2+}, is an important molecular mechanism modulating intracellular Ca^{2+} signaling (Bi et al., 2013; Faouzi et al., 2016; Funabashi et al., 2010; Si et al., 2006). We investigated whether the IK_{Ca} channel is involved in substrate stiffness regulation of the $[Ca^{2+}]_i$ in VSMCs. On average, the $[Ca^{2+}]_i$ in VSMCs growing on the stiff substrate was significantly higher than in those on the soft substrate (Fig. 4). Treatment with TRPM34, without effect on the $[Ca^{2+}]_i$ in cells on the soft substrate, largely prevented stiff substrate-induced increase in $[Ca^{2+}]_i$ (Fig. 4). Collectively, these results showed that stiff substrate induces increase in the $[Ca^{2+}]_i$ in an IK_{Ca}-dependent manner.

We next evaluated whether such IK_{Ca}-dependent intracellular Ca^{2+} signaling plays a role in stiff substrate-induced increase in VSMC proliferation. Prolonged inclusion of 2 mM EGTA, a Ca^{2+} chelator, in the cell culture medium resulted in a noticeable detrimental effect manifested by cell detachment from the soft substrate. Treatment with 1 mM EGTA, without significant effect on cell proliferation on the soft substrate, strongly inhibited the increase in cell proliferation on the stiff substrate (Fig. 5A). Similar results were obtained using 10 μM BAPTA-AM to suppress intracellular Ca^{2+} concentration (Fig. 5B), further confirming that IK_{Ca}-dependent intracellular Ca^{2+} signaling is important for stiff substrate-induced increase in VSMC proliferation.

IK_{Ca}-dependent activation of ERK is required for substrate stiffness regulation of cell

proliferation

Finally, we investigated whether activation of the ERK as a Ca^{2+}-dependent downstream signal pathway is engaged in transducing stiff substrate-induced IK_{Ca}-dependent modulation of intracellular Ca^{2+} concentration to cell proliferation. Western blotting showed that stiff substrate induced an increase in the ERK phosphorylation level (Fig. 6A-C). Furthermore, treatment with TRAM34 to inhibit the IK_{Ca} channel (Fig. 6, A and C) or treatment with BAPTA-AM to prevent an increase in intracellular Ca^{2+} concentration (Fig. 6B-C), while having no effect on the ERK activity in cells on the soft substrate, strongly inhibited stiff substrate-induced increase in the ERK phosphorylation. These results clearly indicate stiff substrate-induced IK_{Ca} channel-dependent induction of intracellular Ca^{2+} signalling leads to ERK activation. Finally, treatment with PD98059, an ERK inhibitor, largely prevented stiff substrate-induced increase in cell proliferation (Fig. 6D), strongly supporting that ERK activation as a downstream signal pathway is critical for stiff substrate-induced increase in cell proliferation.

DISCUSSION

In the present study, we show that substrate stiffness of pathological relevance upregulates the IK_{Ca} channel expression in A7r5 cells and stimulates cell proliferation. We further reveal that IK_{Ca}-mediated modulation of intracellular Ca^{2+} signaling and the ERK signal pathway are critical for substrate stiffness regulation of cell proliferation. These findings, despite being from A7r5 cells, a widely used cell model of VSMCs and needing to be verified in primary VMSCs, provide a potential molecular mechanism by which extracellular matrix stiffness under physiological and pathological conditions regulates VSMC proliferation.

The blood vessels such as arteries under the physiological condition have a Young's modulus of 0.2~0.6 MPa and become substantially stiff with ageing or under pathophysiological conditions such as vascular stiffening in cases of atherosclerosis, hypertension and aneurism (Ebrahimi, 2009; Karimi, Navidbakhsh, Shojaei, & Faghihi, 2013; Laurent et al., 2002; O'Rourke et al., 2002). Vascular stiffening is a common feature and causatively associated with the progression of multiple CVDs (Cavalcante et al., 2011; Cecelja & Chowienczyk, 2012; Hoffman et al., 2017; Laurent et al., 2002; Wykretowicz et al., 2009). In this study, we examined A7r5 cells growing on substrates with stiffness from 0.21 to 1.72 MPa, thus covering the range of artery stiffness from physiological to pathological conditions. The first finding from this study is that cells exhibited significantly greater spreading and faster proliferation on pathologically relevant substrate stiffness (Fig. 1). These results are similar to those reported in previous studies using polyacrylamide gels or poly (ethylene glycol) hydrogels-based substrates that show stiff substrate stimulates VSMC proliferation (Brown et al., 2010; Peyton et al., 2006; Xie et al., 2018). Flow-induced shear stress and membrane stretch have also been

shown to stimulate the expression or activity of the IK_{Ca} channel in VSMCs and other cell types (Brakemeier et al., 2003; Hayabuchi et al., 2011; Takai et al., 2013). Our study examining the mRNA and protein expression levels provides consistent evidence to show that stiff substrates upregulate the IK_{Ca} channel expression (Fig. 2). Thus, the present study extends the finding from previous studies that mechanical stimulation is an important factor regulating the IK_{Ca} channel expression and activity. As introduced above, vascular stiffening is a common characteristic of multiple CVDs (Cecelja & Chowienczyk, 2012; Ebrahimi, 2009; Hoffman et al., 2017; Laurent et al., 2002), and VSMC proliferation contributes to the progression of CVDs (Haga et al., 2007; House et al., 2008). Our results show that pharmacological and genetic inhibition of the IK_{Ca} channel largely prevented the increase in cell proliferation induced by substrate with stiffness similar to that of arteries under pathological conditions (Fig. 3). Our findings prompt us to propose upregulation of the IK_{Ca} channel expression and activity as a critical mechanism that links vascular stiffening to VSMC proliferation.

While increasing evidence from studies in various cell types supports an important role of the IK_{Ca} channel in regulating cell proliferation, the underlying mechanism is still obscure. Our study has revealed a novel signaling axis responsible for stiff substrate-induced IK_{Ca} channel-dependent VSMC proliferation. Pathologically relevant matrix stiffness evoked an increase in $[Ca^{2+}]_i$, which was potently inhibited by blockage of the IK_{Ca} channel (Fig. 4). In addition, stiff substrate-induced cell proliferation was largely prevented by reducing extracellular Ca^{2+} concentration using EGTA or an increase in intracellular Ca^{2+} concentration using BAPTA-AM (Fig. 5), supporting that IK_{Ca}-dependent modulation of intracellular Ca^{2+} signaling is critical for substrate stiff regulation of VSMC proliferation. Stiff substrate also induced ERK activation, which was strongly suppressed by

blockage of the IK_{Ca} channel or chelating intracellular Ca^{2+} concentration (Fig. 6A-D). Furthermore, pharmacological inhibition of ERK activation largely prevented stiff substrate-induced increase in cell proliferation (Fig. 6D). Collectively, these results support the notion that pathologically relevant substrate stiffness stimulates cell proliferation via upregulation of the IK_{Ca} channel expression and activity that leads to an increase in $[Ca^{2+}]_i$ and activation of the ERK signalling pathway . Further investigations are required to elucidate the Ca^{2+}-permeable channels that mediate Ca^{2+} influx that is modulated by the IK_{Ca} channel activity. TRPC channels are attractive candidates, as has been demonstrated in human breast cancer cells, in which the IK_{Ca} channel works in concerted action with the TRPC1 channel in determining Ca^{2+} signalling to regulate cell proliferation (Faouzi et al., 2016).

In summary, the present study shows upregulation of the IK_{Ca} channel by pathologically relevant matrix stiffness as a novel mechanism mediating stimulation of VSMC proliferation and defines IK_{Ca} channel-dependent modulation of intracellular Ca^{2+} signalling and ERK as a new signaling pathway responsible for matrix stiffness-induced increase in VSMC proliferation. Such a finding should be helpful in understanding the mechanism by which vascular stiffening regulates VSMC function.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Conception and design: Yubo Fan, Yan Sun and Xiaoling Jia designed the study and wrote the manuscript. Xiaoling Jia, Qingmao Yang, Xinlan Chen, Hao Su, Chao Gao, Yanan Li, Shuwen Zhang, Yufan Zheng, Ziyu Wang and Haikun Wang performed experiments, analyzed and interpreted data. Lin-Hua Jiang participated in data interpretation and revised the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

FIGURE 1 Stiff substrates stimulate A7r5 VSMC spreading and cell proliferation. (A) Mean stiffness of PDMS substrates produced from 5 independent preparations. (B) Representative AFM images of the surface topography of PDMS substrates. (C) Representative fluorescent image of F-actin and spreading of cells cultured on indicated PDMS substrates for 24 h. Red, F-actin staining; blue, nuclear staining. Mean data were from 10-39 cells. $^{**}P < 0.01$ compared to cells on 0.21 MPa. (D) Summary of cell proliferation after cultured on indicated PDMS substrates for 1, 2, 4 and 6 days, from 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

FIGURE 2 Stiff substrates upregulate the IK_{Ca} channel expression in A7r5 VSMCs. A and B Representative images showing the IK_{Ca} mRNA expression using RT-PCR and IK_{Ca} protein cell surface expression using flow cytometry. **C** Summary of the mean data from 4 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

FIGURE 3 Stiff substrate-induced IK_{Ca} channel up-regulation is required for increased A7r5 VSMC proliferation. (A) Summary of the effects of treatment with 100 nM TRAM34 on stiff substrate-induced cell proliferation from 3 independent experiments. (B) Representative western blots showing siRNA-mediated knockdown of the IK_{Ca} protein expression (top) and summary of the data from 5 independent experiments (bottom). (C) Summary of stiff substrate-induced cell proliferation in siRNA-transfected cells from 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared

between 0.21 MPa and 1.72 MPa without any treatment or compared between 1.72 MPa with or without treatment with TRAM34, respectively.

FIGURE 4 Stiff substrate induces IK_{Ca}-dependent increase in $[Ca^{2+}]_i$ in A7r5 VSMCs. (A) Representative single cell images showing intracellular Ca^{2+} concentration in cells cultured on the soft and stiff substrate under indicated conditions. Treatment with 100 nM TRAM34 was made during culture media and also in extracellular recording solution. (B) Summary of the relative Fluo4 fluorescence intensity, indicative of the $[Ca^{2+}]_i$, in individual cells under the condition shown in (A), with 375 cells examined for each case. ** $P < 0.01$.

FIGURE 5 Stiff substrate-induced IK_{Ca} channel-dependent increases in $[Ca^{2+}]_i$ is essential for increased A7r5 VSMC proliferation. Summary of cell proliferation on substrates in culture media containing 1 mM EGTA (A) or 10 μM BAPTA-AM (B), from 3 independent experiments for each case. **$P < 0.01$.

FIGURE 6 Stiff substrate-induced ERK activation is required for increased A7r5 VSMC proliferation. Representative western blots showing stiff substrate-induced ERK activation in cells without or with treatment with 100 nM TRAM-34 (A) or in culture media containing no or 10 μM BAPTA-AM (B). (C) Summary of the mean data from 3 independent experiments. (D) Summary of the effects of treatment with 20 μM PD98059 on stiff substrate-induced cell proliferation from 3 independent experiments. *$P < 0.05$ and **$P < 0.01$.

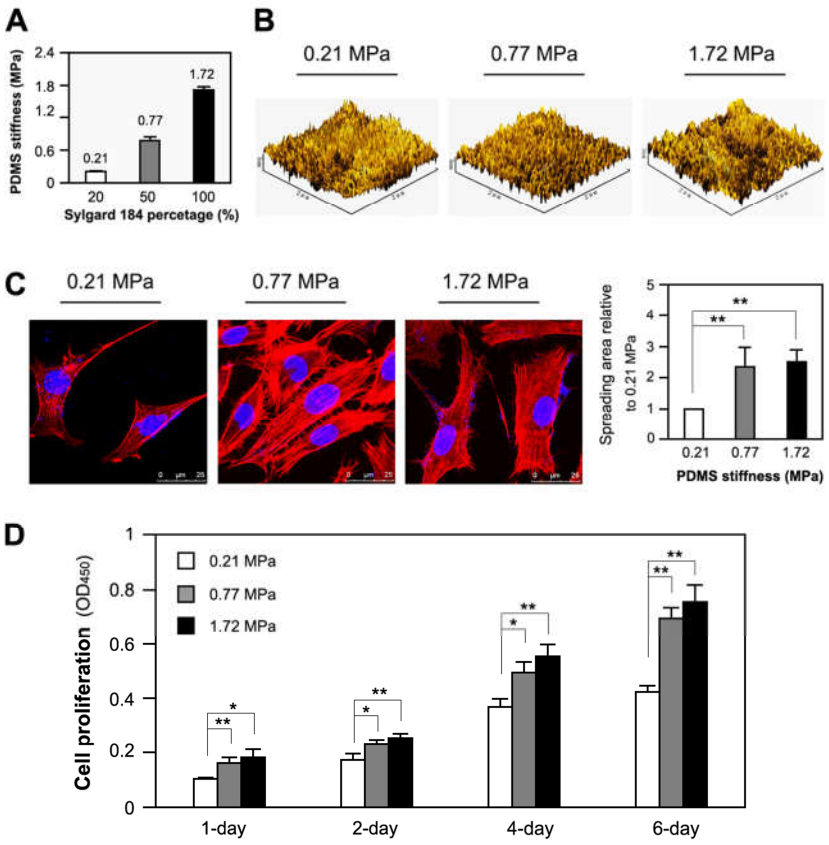
A
PDMS stiffness (MPa)
2.4
1.8
1.2
0.6
0
0.21
0.77
1.72
20
50
100
Sylgard 184 percetage (%)
B
0.21 MPa
0.77 MPa
1.72 MPa
C
0.21 MPa
0.77 MPa
1.72 MPa
Spreading area relative to 0.21 MPa
5
4
3
2
1
0
**
**
0.21
0.77
1.72
PDMS stiffness (MPa)
D
Cell proliferation (OD450)
1
0.8
0.6
0.4
0.2
0
0.21 MPa
0.77 MPa
1.72 MPa
*
**
**
*
**
*
**
**
1-day
2-day
4-day
6-day

FIGURE 1

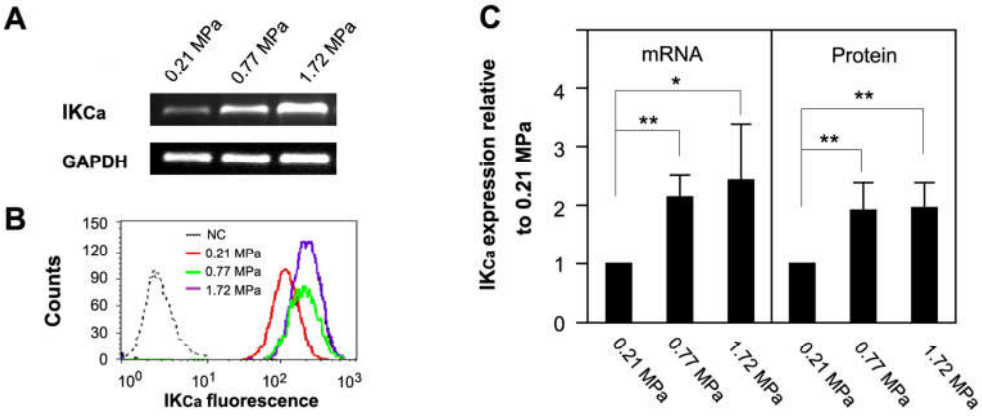
A
0.21 MPa
0.77 MPa
1.72 MPa
IKCa
GAPDH
B
Counts
NC
0.21 MPa
0.77 MPa
1.72 MPa
IKCa fluorescence
C
IKCa expression relative to 0.21 MPa
mRNA
Protein
*
**
**
**
0.21 MPa
0.77 MPa
1.72 MPa
0.21 MPa
0.77 MPa
1.72 MPa

FIGURE 2

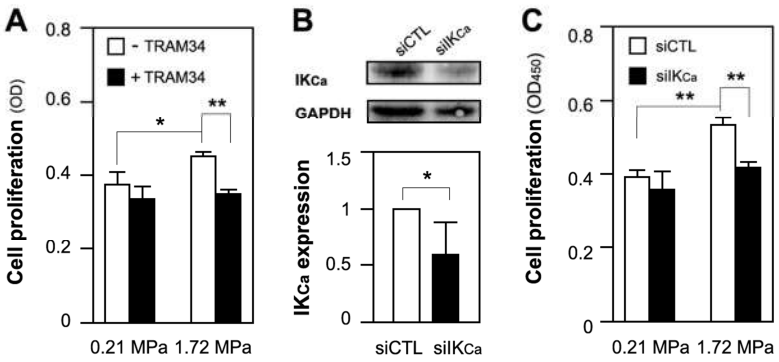
A
B
C
- TRAM34
+ TRAM34
Cell proliferation (OD)
0.21 MPa 1.72 MPa
siCTL
siIKCa
IKCa
GAPDH
IKCa expression
siCTL siIKCa
Cell proliferation (OD450)

FIGURE 3

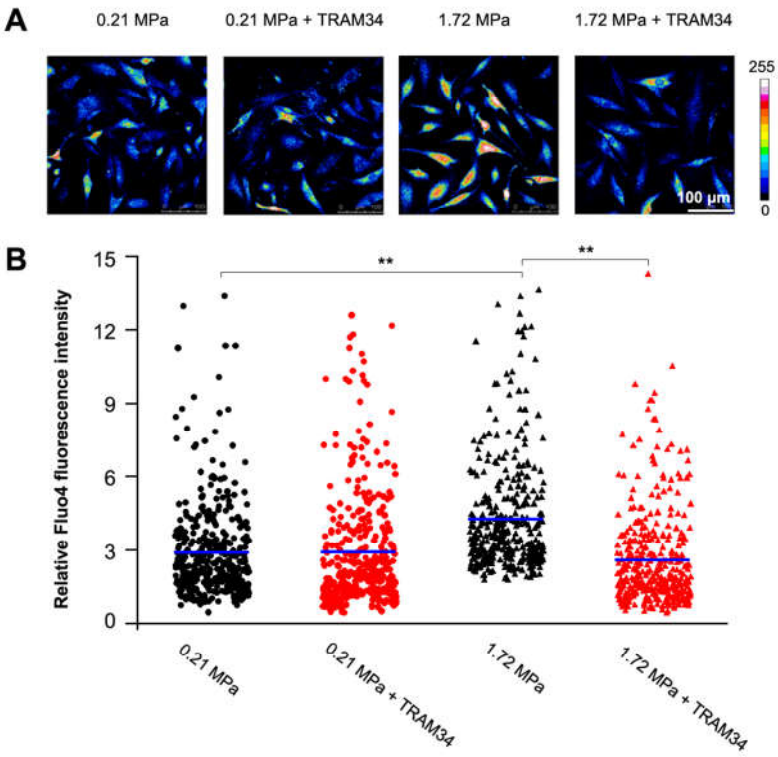
A
0.21 MPa
0.21 MPa + TRAM34
1.72 MPa
1.72 MPa + TRAM34
255
0
100 μm
B
Relative Fluo4 fluorescence intensity
15
12
9
6
3
0
**
**
0.21 MPa
0.21 MPa + TRAM34
1.72 MPa
1.72 MPa + TRAM34

FIGURE 4

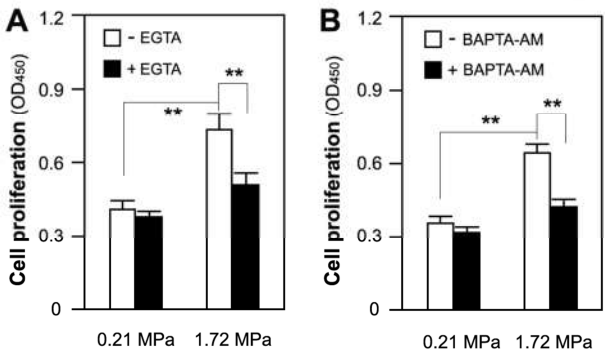
A
B
- EGTA
+ EGTA
- BAPTA-AM
+ BAPTA-AM
Cell proliferation (OD450)
1.2
0.9
0.6
0.3
0
**
0.21 MPa
1.72 MPa

FIGURE 5

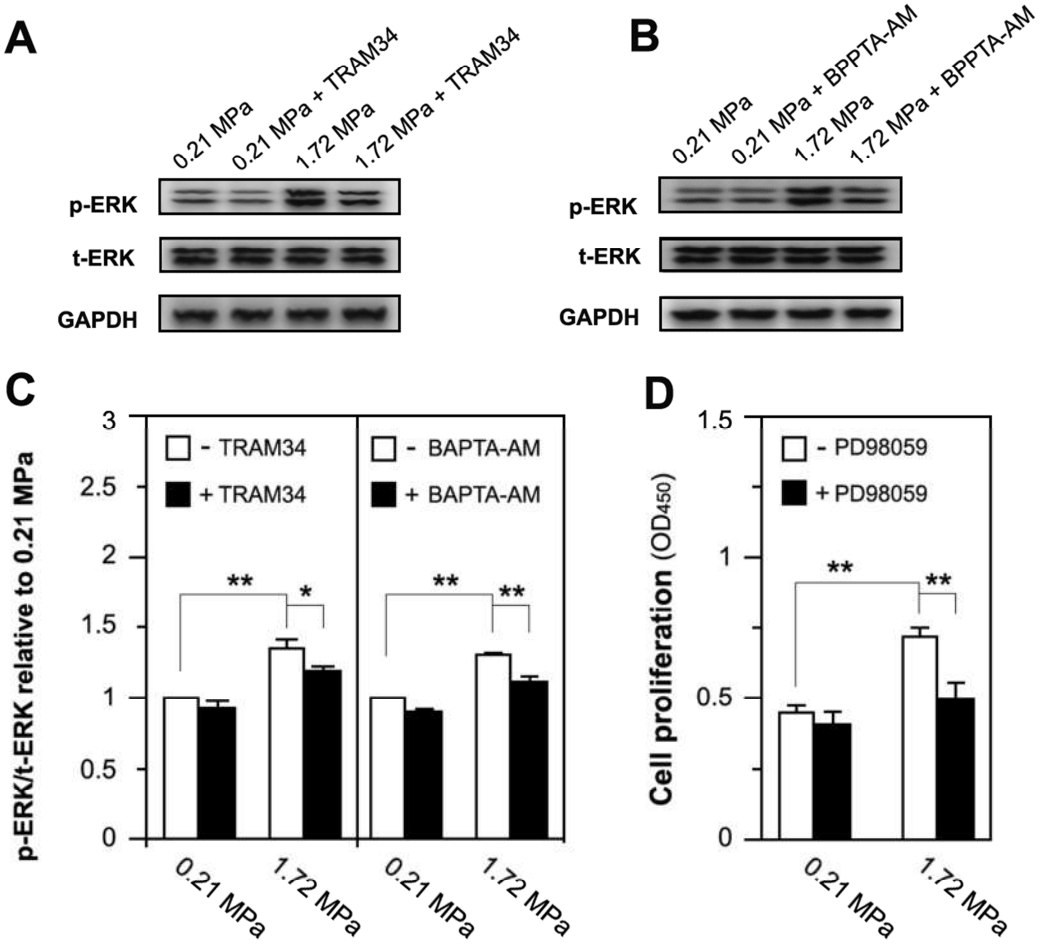
A
0.21 MPa
0.21 MPa + TRAM34
1.72 MPa
1.72 MPa + TRAM34
p-ERK
t-ERK
GAPDH
B
0.21 MPa
0.21 MPa + BPPTA-AM
1.72 MPa
1.72 MPa + BPPTA-AM
p-ERK
t-ERK
GAPDH
C
p-ERK/t-ERK relative to 0.21 MPa
- TRAM34
+ TRAM34
- BAPTA-AM
+ BAPTA-AM
0.21 MPa
1.72 MPa
0.21 MPa
1.72 MPa
D
Cell proliferation (OD450)
- PD98059
+ PD98059
0.21 MPa
1.72 MPa

FIGURE 6