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Visualization of stimulus-specific heterogeneous activation of individual vascular smooth muscle cells in aortic tissues

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Abstract

Intercellular communication among autonomic nerves, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) plays a central role in an uninterrupted regulation of blood flow through vascular contractile machinery. Impairment of this communication is linked to development of vascular diseases such as hypertension, cerebral/coronary vasospasms, aortic aneurism, and erectile dysfunction. Although the basic concept of the communication as a whole has been studied, the spatiotemporal correlation of ECs/VSMCs in tissues at the cellular level is unknown. Here we show a unique VSMC response to ECs during contraction and relaxation of isolated aorta tissues through visualization of spatiotemporal activation patterns of smooth muscle myosin II. ECs in the intimal layer dictate the stimulus-specific heterogeneous activation pattern of myosin II in VSMCs within distinct medial layers. Myosin light chain (MLC) phosphorylation (active form of myosin II) gradually increases towards outer layers (approximately 3 fold higher MLC phosphorylation at the outermost layer than that of the innermost layer), presumably by release of an intercellular messenger, nitric oxide (NO). Our study also demonstrates that the MLC phosphorylation at the outermost layer in spontaneously hypertensive rats (SHR) during NO-induced relaxation is quite high and approximately 10 fold higher than that of its counterpart, the Wistar-Kyoto rats (WKY), suggesting that the distinct pattern of myosin II activation within tissues is important for vascular protection against elevated blood pressure.

Keywords

myosin; vascular smooth muscle cells; endothelial cells; hypertension; vascular injury

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Contributions

S.K. and T.K. shared to design and conduct all of the experiments, data analysis, and draft the manuscript. M.I. directed the project and discussed with other authors and helped to develop the final version of the manuscript.

Competing financial interestss

The authors declare no competing financial interests.

Introduction

Contraction of the vascular smooth muscle cell (VSMC) is initiated by phosphorylation of regulatory myosin light chain (MLC), which is controlled by the balance of enzymatic activity between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP)(Somlyo and Somlyo, 1994; Stull et al., 1991). Consequently, the amount of MLC phosphorylation represents the degree of VSMC activation. In response to contractile external stimuli, cytosolic Ca^{2+} in smooth muscle is increased through both Ca^{2+} release from the intracellular stores (sarcoplasmic reticulum; SR) and entry of the extracellular Ca^{2+} via the plasma membrane Ca^{2+} channels. The Ca^{2+} binds calmodulin (CaM) and activates Ca^{2+} /CaM-dependent MLCK, which phosphorylates MLC(Khalil, 2010). In contrast, smooth muscle relaxation initially occurs by a decreased intracellular Ca^{2+} concentration that reduces MLCK activity through the dissociation of Ca^{2+} /CaM complex, and MLCP dephosphorylates phosphorylated MLC(Khalil, 2010; Webb, 2003).

On the other hand, it is well known in systemic circulation that sympathetic nerves through alpha1 agonist positively regulate and endothelial cells (ECs) through nitric oxide (NO) negatively regulate MLC phosphorylation and VSMC contraction(Somlyo and Somlyo, 1994; Stull et al., 1991). The former agonist induces both the activation of MLCK through the increase of intracellular Ca^{2+} concentration and the inhibition of MLCP through activation of G protein-coupled both PKC/CPI-17 and RhoA/ROCK pathways (so-called Ca^{2+} sensitization)(Somlyo and Somlyo, 2003; Stull et al., 1991). In contrast, the latter NO gas leads to an increase in cGMP and the subsequent activation of cGMP-dependent protein kinase (PKG) signaling that promotes vasodilation by both the deactivation of the MLCK via a decrease in intracellular Ca^{2+} concentration(Cohen et al., 1999; Plane et al., 1998; Sanders et al., 2000) and deinhibition of MLCP via an inhibition of Ca^{2+} sensitizing pathways(Kitazawa et al., 2009; Sauzeau et al., 2000; Wu et al., 1996). Therefore, it is well established that impairment of the communication between ECs and VSMCs leads to an increase in smooth muscle contractility and development of cardiovascular diseases(Feletou et al., 2009). The loss of endothelial integrity is associated with a reduction in NO bioavailability and thus correlated with the impairment of vasodilatation(Deanfield et al., 2007; Sena et al., 2013).

Large blood vessels are composed of three layers: endothelial monolayer (tunica intima), multiple smooth muscle layers (tunica media) and connective tissue (tunica externa). Each layer is delimited by internal elastic lamina(Sandow et al., 2009). Previous studies have demonstrated that MLC is maximally phosphorylated approximately 50% of total myosin during agonist-stimulated contraction in blood vessels (Dimopoulos et al., 2007; He et al., 2011; Kitazawa and Kitazawa, 2012a) and other smooth muscle tissues under physiological conditions (He et al., 2008; Tsai et al., 2014; Zhang et al., 2010). Despite extensive studies, however, it remains to be elucidated under what conditions for individual cells is the half-maximally active form of VSMCs in tunica media, how ECs regulate it, and how it contributes to the pathogenesis and pathophysiology of vascular diseases such as hypertension, vascular injury, etc. Characterization of the complex communication of ECs/VSMCs in vascular tissue is therefore an important challenge.

In the present study, we developed a novel imaging method to observe contractile activation of individual VSMCs in tissues by monitoring each MLC phosphorylation, which is coupled with tissue contractility. Using this technique, we demonstrated VSMCs throughout the tunica media to assess the spatiotemporal changes in the VSMC activity under the influence of ECs and, furthermore, succeeded in observing the different contractile responses under pathological situations such as vascular injury/inflammation and hypertension (Furchgott and Zawadzki, 1980; Schwartz et al., 1980; Simon, 2012; Tanaka et al., 1993).

Materials and Methods

Antibodies

Anti-pMLC Ab specifically recognized the mono-phosphorylated MLC at Ser19 (Komatsu et al., 2000). Anti-MLC, RhoA, PKC α , PKG1 α , ROCK α , PKG1 β , and ROCK β antibodies (Abs) were purchased from Santa Cruz Biotechnology. Anti-smMHC Abs were purchased from abcam, MLCK from Pierce, MYPT1 (myosin phosphatase target subunit 1) from Millipore, and α -actin from Sigma.

Tissues preparations for measuring of contraction and quick freezing

All animal procedures were approved by the Animal Care and Use Committee of the University of Texas Health Science Center at Tyler (Permit Number: 542). Male Wister rats of 250–350 g weight and WKY and SHR both 12 weeks old were killed with CO₂ gas inhalation. The CO₂ method is recommended for small animals by the American Veterinary Medical Association (AVMA) Guidelines of Euthanasia. To confirm their death, the cervical dislocation was performed. All methods were performed in accordance with the relevant guidelines and regulations. It is known that the major increase in blood pressure of SHR occurs until the 12th week of age although either cGMP or cAMP-dependent phosphodiesterase activity is still not increased yet (Bucher and Stoclet, 1980; Mulvany et al., 1980). After thoracotomy, the thoracic aortas were isolated. Following dissection of fat and soft connective tissues in tunica adventitia with a small piece of razor blade, each aorta segment was cut into rings of 1.5 mm in length. In some of the rings, endothelial layers were removed using gentle rubbing with a razor blade (Furchgott and Zawadzki, 1980). One side of each U-shaped 0.1 mm-diameter tungsten clip was inserted into the arterial ring as previously reported (Kitazawa and Kitazawa, 2012b). One side of the clip outside the ring was tied with a monofilament silk thread, which was hooked to a tungsten extension needle connected to a force transducer (AM801, SensoNor, Horten, Norway), and the other side to a micromanipulator. The length of arterial ring segment was adjusted to produce a maximum force as previously described (Dimopoulos et al., 2007).

Normal external solution was 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 5.6 mM glucose, 30 μ M EDTA and 5 mM Hepes. For the depolarizing high K⁺ solution, 120 mM KCl was substituted for the same molar concentration of NaCl to make up total 124 mM KCl. Both solutions were adjusted to pH 7.4 with Tris. The solution temperature was maintained at 35°C throughout the experiments. Each aorta ring was repeatedly stimulated for 3 min with 124 mM K⁺ solution at 15 min intervals until the peak contraction within 3 min no longer increased. The rings were then alternately stimulated with high K⁺ and 10 μ M

phenylephrine (PE) until the PE-induced contraction no longer increased. PE concentrations at 10 μM produced a maximum contraction, which was no longer increased by higher concentrations of PE. Treatment with high K^+ between the PE-induced contractions was required to maintain constant SR Ca^{2+} loading along with a reproducible time course and amplitude of PE-induced contractions (Dimopoulos et al., 2007).

Tissue preparations for Alkali-urea-glycerol gel electrophoresis

To measure the phosphorylation levels of MLC using Alkali-urea-glycerol (Urea-glycerol) gel, four conditions in aorta were employed: the aortas were quick-frozen by plunging into liquid nitrogen-cooled propane ($<-150^\circ\text{C}$) (1) at rest, (2) 1 min after stimulation with 10 μM PE, (3) 1 min after stimulation with 10 μM PE in the presence of 1 μM ACh pre-applied 4 min before PE, and (4) 1 min after stimulation with 10 μM PE in the presence of 1 μM ACh and 100 μM L-NAME pre-applied 15 min before PE + ACh.

For the Urea-glycerol gel electrophoresis, quick-frozen samples were transferred onto liquid N_2 -cooled 10% TCA-acetone and incubated overnight at -80°C . After gradually warmed up to room temperature, the samples were rinsed with acetone three times, dried under vacuum, and kept at -80°C for later use. The dried samples were homogenized in 10% TCA in water. The powdered samples were rinsed with acetone and homogenized in the warm urea solution. In some experiments, the medial part of aorta was split into halves to separately measure the amount of phosphorylated MLC in inner and outer layers of the media as follows. First, dried aortic rings were circularly cut by about 100 μm in thickness (Supplementary Fig. S1A). The thin rings were incubated in H_2O for maceration and cut open. Middle of the medial layer at the edge of aorta strips was incised by a small razor blade and split into halves by fine forceps (Supplementary Fig. S1B). The thread-like aorta strips were separately collected for each layer, dried, and homogenized in 10% TCA in water as described above.

To quantify the phosphorylation level of MLC, we have performed Urea-glycerol gel electrophoresis analysis (Perrie and Perry, 1970). Urea-glycerol gel allowed us to separate the different phosphorylation states of MLC among unphosphorylated MLC, monophosphorylated MLC and diphosphorylated MLC according to the difference in the charges of each phosphorylation state of MLC. After the separation of the different phosphorylation states of MLC by Urea-glycerol gel electrophoresis, the MLC bands were transferred from the Urea-glycerol gel to nitrocellulose membrane for western blot detection using a pan-MLC antibody (Santa Cruz Biotechnology, sc-15370) that recognized MLC in both unphosphorylated and phosphorylated MLCs (Komatsu and Ikebe, 2004; Komatsu and Ikebe, 2007; Komatsu et al., 2000). We calculated the % MLC phosphorylation by dividing the signal of monophosphorylated MLC band by that of total MLC bands (the position at the upper band (0-p) indicates unphosphorylated MLC band and the position at the lower band (1-p) indicates monophosphorylated MLC band). It should be noted that we have detected unphosphorylated MLC (0-p) and monophosphorylated MLC (1-p) in the present conditions, but not diphosphorylated one. It is also noted that the Urea-glycerol gel electrophoresis should exhibit thicker (or broader) bands than the conventional SDS-PAGE, because of the different principles of those gel electrophoresis analyses. The SDS-PAGE is able to

concentrate the samples within stacking gel and then separate the bands according to the different molecular weights, while Urea-glycerol gel does not have the stacking gel and separates MLCs according to the difference in the charges of each phosphorylation state of MLC.

Western blot analysis was performed as described previously (Komatsu and Ikebe, 2004; Komatsu and Ikebe, 2007; Komatsu et al., 2000). The amount of proteins was determined by scanning densitometry (BIO-RAD ChemiDoc XRS+ Imaging system) using NIH image program (Komatsu and Ikebe, 2004; Komatsu and Ikebe, 2014).

Immunohistochemical analysis

For immunohistochemical analysis, samples quick-frozen under various conditions were transferred onto liquid N₂-cooled acetone containing 0.1 μ M calyculin A and 0.1 μ M staurosporine and incubated overnight at -80°C to remove H₂O and block both protein kinase and phosphatase activities. After gradually warmed up to room temperature, the samples were fixed with 4% formaldehyde in PBS containing calyculin A as a phosphatase inhibitor, staurosporine as a kinase inhibitor, 2 mM MgCl₂ and 1 mM EGTA at room temperature for 20 min. After rinsed with PBS 3 times, the aorta rings were embedded in warmed 4% agar and cooled down on ice. The agar-embedded samples were sectioned in ice cooled PBS by EMS OTS-4000 (Precision Instruments Inc., Greenville, NC, USA) at 110 μ m in thickness. The sectioned samples were removed from the agar, treated with 0.1 % Triton X-100 in PBS, and rinsed with PBS 3 times. After incubated in the blocking PBS solution containing 1% BSA, the sections were placed in PBS containing paired primary antibodies (pMLC and smMHC Abs) and incubated in a humidified box overnight at 4°C . The unbound primary antibodies were removed thrice with PBS. The samples were incubated with secondary Abs conjugated with a fluorescence dye (Alexa Fluor-555 conjugated anti-mouse and Alexa Fluor-647 conjugated anti-rabbit Abs) (Life Technologies) and, in addition, Cytogreen for staining DNA for 90 min at RT. After washed with PBS, the sections were immersed by antifading agent (1, 4-Diazobicyclo-(2,2,2-octane (DABCO)).

Image Processing

Differential interference contrast (DIC) and fluorescence images were obtained using a Leica TSC SP8 confocal laser scanning microscopy system (Leica Microsystems Inc., Heidelberg, Germany). A series of optical sections were collected at 1 μ m intervals in Z-axis (15–20 μ m). These multiple Z-series sections were projected onto one plane. Using LCS 3D software of Leica Microsystems, fluorescence ratio analyses were performed to obtain the signal intensity ratio image (SIRp) between pMLC and smMHC, and the length of distance in the images were measured (Komatsu and Ikebe, 2004; Komatsu and Ikebe, 2014).

Statistical analysis

Data were expressed as mean $\pm$ a standard error of the mean (s.e.m). Statistical significance was tested with a two-tailed Student's t-test for comparing between two groups, a one-way ANOVA followed by the Tukey-Kramer test for comparing among multiple groups. The difference was considered to be significant if p was <0.05 .

Results

Effect of external stimuli on contraction and MLC phosphorylation in arterial smooth muscle tissue

We first quantified the amount of phosphorylated MLC (pMLC) during various contractile states to evaluate the corresponding smooth muscle myosin II activity in rat aorta (Fig. 1), by a combination of force measurement technique and an established method to determine the phosphorylation level of MLC (Urea-glycerol gel electrophoresis)(see Methods for details). At 60 sec after $\alpha 1$ agonist phenylephrine (PE) stimulation, a contraction of the EC-intact vessel was increased to $70 \pm 3\%$ ($n=6$) of control KCl-induced contraction (Fig. 1A upper graph, I-PE, red line). PE induced contraction was accompanied by simultaneously elevated pMLC by $47 \pm 4\%$ of total MLC (Fig 1B, lane 2). In all EC-intact aortic vessels used in this study, an administration of $1 \mu\text{M}$ acetylcholine (ACh) reduced the PE-induced contraction to less than 25% of PE alone within 2 min, whereas the removal of EC layer completely abolished the ACh-induced relaxation (Fig 1C)(Furchgott and Zawadzki, 1980). In contrast, a 4 min pretreatment with ACh suppressed the PE-induced development of contraction to $30 \pm 4\%$ (Fig. 1A upper graph, I-ACh, blue line) and reduced pMLC to $4.3 \pm 1\%$ (Fig. 1B, lane 3)(Furchgott and Zawadzki, 1980). Either administration of L-NAME, a nitric oxide synthase (NOS) inhibitor(Pfeiffer et al., 1996), or intimal denudation eliminated the inhibitory effect of ACh on both contraction (Fig. 1A, upper graph, green line and lower graph, blue line, respectively) and pMLC to a level similar to that of PE alone (Fig. 1B, lanes 2 vs. 4, $47 \pm 4\%$ vs. $52 \pm 5\%$). Together, these confirm that the relaxing effect of ACh in the aortic VSMCs is mediated through NO gas from the ECs(Griffith et al., 1984; Kitazawa et al., 2009).

Possible models for spatial distribution of measured pMLC in VSMCs throughout tunica media during agonist-stimulated contraction are as follows: 1) Even model; all VSMCs with 50% of pMLC throughout the tunic media. 2) On/Off model; half of VSMCs having 100% pMLC and the other half 0%. 3) Random model; VSMCs with different levels of pMLC (0~100%) throughout the tunica media in a random manner. 4) Gradient model; VSMCs with pMLC of different levels (0~100%) sequentially distributed in the tunic media. All models yield about 50% of pMLC in total. Herein, we demonstrate which is the most suitable model in aortic tissues.

Spatiotemporal change in individual smooth muscle cell activity in aorta tissues by external stimuli

We investigated the stimulus-specific spatiotemporal patterns of pMLC in VSMCs of PE-stimulated rat aorta by a combination of force measurement, quick freezing and imaging techniques (see Methods for details). Fig. 2A shows each representative confocal image of pMLC (upper left panel, red), smooth muscle myosin heavy chain (smMHC, upper right, cyan), nuclei (lower left, green) and differential interference contrast (DIC) of EC-intact aorta at 60 sec after PE stimulation (see Methods for details). To quantify pMLC levels in images, a signal intensity ratio image (SIRp) between pMLC and smMHC was acquired by dividing the signal intensity of pMLC by that of smMHC (Fig. 2B). Interestingly, SIRp, the quantified pMLC, gradually increased from the inner to outer layers of tunica media (Fig.

2B). To show this uneven distribution more clearly, the two-dimensional SIRp were longitudinally summed up along the length of the media and averaged among 7 independent aorta rings (Fig. 2B, lower panel). The resultant figure clearly indicates a progressive change in pMLC, low in the inner near the intima and high in the outer periphery (Fig. 2B, lower panel, 0.288 ± 0.075 in the innermost layer vs. 0.875 ± 0.125 in the outermost layer). The average values of SIPp in outer half layers was about two times higher than that in inner half layers (Fig. 2C, I-PE inside vs. I-PE outside, 0.2573 ± 0.03 vs. 0.6057 ± 0.06). It should be noted that the spike-like fluctuation of SIRp was mainly due to location of elastin layers between the VSMCs layers. The observed image of Fig. 2B agrees with the gradient model, showing that the extent of pMLC of *in-situ* VSMCs stimulated with a given concentration of α_1 agonist is lower in VSMCs at the smooth muscle layers closer to the EC layer. It further suggests an effect of NO released from ECs on VSMC contraction even when the ECs were not stimulated (Ohno et al., 1993).

To verify whether the gradient model for VSMCs is due to the function of ECs, ACh was used to stimulate the NO release from ECs. The SIRp was notably lower in the presence of ACh than its absence throughout the tunica media and, in particular, more suppressed in VSMCs closer to the intima (Fig. 3A, I-ACh). In contrast, L-NAME, NO synthase inhibitor, in the presence of ACh neutralized the effect of ACh and furthermore reduced the basic gradient pattern (Fig. 3A, I-LNAME; Fig. 3C, left graph, I-PE inside vs. I-LNAME inside, 0.257 ± 0.03 vs. 0.445 ± 0.06), suggesting that the gradient pattern of SIRp is mainly due to the NO diffusion from the intima. Likewise, the physical EC-denudation eliminated the basic gradient pattern of SIRp and reduced the influence of ACh (Fig. 3B, D-PE and D-ACh; also see Fig. 3C, middle graph, I-PE inside vs. D-PE inside, 0.257 ± 0.03 vs. 0.590 ± 0.03 ; right graph, I-ACh inside vs. D-ACh inside, 0.0833 ± 0.02 vs. 0.463 ± 0.04 , I-ACh outside vs. D-ACh outside, 0.205 ± 0.04 vs. 0.638 ± 0.056). These together suggest that the gradient is primarily due to NO release from ECs but neither due to slower diffusion of PE from the intima than from the periphery nor a higher sensitivity of the peripheral VSMCs to α_1 agonist.

To further validate the gradient model supported by the imaging analyses, we separately determined the amount of pMLC of VSMCs in the outer and inner layers of the tunica media using the conventional biochemical approach (Urea-glycerol gel electrophoresis) as shown in Fig. 1B. PE-stimulated EC-intact and denuded aorta tissues were mechanically split into the inner and outer layers (see the Methods for details). In the EC-intact aorta, pMLC stimulated with PE alone was modestly lower in the inner layers than that of outer layers (Fig. 4A, lanes 1 vs. 2, $35 \pm 4\%$ vs. $55 \pm 4\%$), similar to the SIRp of Fig. 2B. Pretreatment with ACh almost totally reduced pMLC in the inner layers (Fig. 4A, lane 3, $1.4 \pm 1\%$), whereas pMLC in the outer layers only partially reduced (Fig. 4A, lane 4, $29 \pm 5\%$), which agrees with the image of the middle panel of Fig. 3A (I-ACh). The EC-denudation abolished the difference in pMLC between inner and outer layers (Fig. 4A, lanes 5 vs. 6, $51 \pm 4\%$ vs. $56 \pm 5\%$) regardless of the presence of ACh (Fig. 4A, lanes 7 vs. 8, $49 \pm 6\%$ vs. $57 \pm 7\%$). These biochemical results are consistent with the imaging analyses data in Figures 2 and 3. As shown in Fig. 4B, there was no obvious difference between the inner and outer layers of tunica media in the expression level of various SM proteins that play key roles in the regulation of arterial pMLC. It should be noted that SM specific MHC (smMHC), and α -

actin (specific for SMCs, also loading control of SMC contents in both layers) and MLC, of which the expression level should be proportional to MHC, were detectable in similar amounts within both inner and outer layers, indicating that the amount of MLC as well as other proteins in non-muscle cells within adventitia (un-removal tight connective tissue; see the Methods) is negligible in the outer medial layers. This result supports the idea that the gradual change in pMLC levels in tunica media results from the NO release from the intima.

Spatiotemporal analysis of individual smooth muscle cell activity in pathological conditions

We next analyzed the importance of this spatiotemporal change in VSMC activation regarding the spatial location between ECs and VSMCs under pathological conditions, which can hardly be analyzed by conventional biochemical techniques. To obtain a clue about what happens in the VSMCs following local mechanical injury of endothelium such as balloon angioplasty and endovascular stent deployment (Furchgott and Zawadzki, 1980; Schwartz et al., 1980; Simon, 2012; Tanaka et al., 1993), ECs were partially removed to study the effect of local EC damage on the VSMC activation in tunica media (Fig. 5). As shown in Fig. 5A (bottom panel, merged image of nuclei and smMHC), the nuclei of VSMCs in medial layers showed an elongated shape that is quite different from the round shape of nuclei of the intimal layer (endothelial cells), which are located outside of the VSMC layers (Fig. 5A, bottom panel, double-head arrow area according to the EC nuclei). These results indicate that the left half of the region was denuded. As expected, the SIRp in the EC-denuded area of the media remained relatively high, whereas that of the EC-intact area (Fig. 5A, double-head arrow area according to the EC nuclei) appeared to be gradually reduced away from the denuded area (Fig. 5, B and C), indicating uneven VSMC contractions localized in a circumferential vessel. Consistent with this view, ACh-induced relaxation of the half-denuded aorta was considerably less than that of the EC-intact one (Fig. 5D, red line vs. blue line). The relative force of the PE-stimulated half-denuded aorta was approximately 50% of the EC-denuded one at 3 min (total 5 min) after the administration of ACh, and the EC-denudation of the entire EC layer completely abolished the ACh-induced relaxation (Fig. 5D, black line).

For another pathological model, we examined young (12 weeks old) spontaneously hypertensive rats (SHR), which were compared to age-matched counterpart Wistar-Kyoto rats (WKY), and found a unique VSMC response to ACh-stimulated ECs in this disease model. Maximal stimulation with PE in SHR resulted in the gradual distribution of SIRp similar to that of WKY (Fig. 6, A and B), consistent with the result of no difference in the force developments between the two models (Fig. 6C). In the presence of ACh, however, the imaging analyses revealed markedly high SIRp at only the outermost layer of SHR VSMCs stimulated with PE, while other large areas, including intimal to middle VSMC layers, in SHR were dark showing little SIRp similar to that of the whole area in WKY (Fig. 6D). Mechanical measurements under the same conditions found slightly higher contraction with mechanical oscillations in SHR than in WKY (Fig. 6F). The result agrees with the difference in the SIRp at the outermost layer between SHR and WKY (Fig. 6E, black line vs. red line, 1.175 ± 0.225 in SHR vs. 0.088 ± 0.038 in WKY). Consistent with the imaging analyses data in Fig. 6A, pMLC levels in the inner and outer layers of both WKY and SHR were

different (Fig. 7A, lanes 1 vs. 2 and 3 vs. 4). Although pMLC was substantially reduced by the administration of ACh, the level of pMLC in the outer layers in SHR was significantly higher than that in WKY (Fig. 7B, lanes 2 vs. 4), which agrees with the image of Fig. 6D.

The difference in the ACh dependent decreases in pMLC is not due to ACh affinity, since SHR and WKY displayed similar sensitivities to ACh for relaxation (Fig. 8A). On the other hand, the thickness of SHR tunica media was significantly larger than that of WKY (Fig. 8B, $86.37 \pm 1.92 \mu\text{m}$, $n=25$ in WKY vs. $93.51 \pm 1.36 \mu\text{m}$, $n=41$ in SHR, $p<0.005$) without increasing the number of the smooth muscle layers (7.06 ± 0.15 in WKY vs. 7.06 ± 0.09 in SHR). It should be noted that the distance from peaks of SIRp between inner and outer smooth muscle layers in SHR was not significantly different from that in WKY ($82.47 \pm 7.84 \mu\text{m}$, $n=7$ in WKY vs. $85.66 \pm 9.72 \mu\text{m}$, $n=7$ in SHR).

Discussion

In the present study, we first provided direct evidence that the activation of smooth muscle cell contraction is dependent on the location of these cells from ECs in aortic intima, which dictate location-dependent activation of individual VSMCs in the medial layers. Using imaging technique, we unveiled the intercellular distribution of the half-maximal activation of VSMCs during agonist-stimulated contraction in aortic tissue. Our results showed that NO released from ECs regulates the activation level of VSMCs depending on the physical location of smooth muscle cells within tunica media. It is reasonable that the force generated by VSMCs is higher in the outer and lower in the inner layers to compact the tunica media, which is in well agreement with Laplace's Law (the tension (T) in each of the muscle layers is directly proportional to the transmural pressure (P) and the radius (r) of the muscle layer, $T=Pr$). This means that smooth muscle cells in the outer layers should generate larger force to maintain the same transmural pressure. Laplace's law applied in the physiology of blood flow and plays an important role in explanations of the wall tension of blood vessels and the behavior of aneurisms (Bisford, 2002). The differential activation of VSMCs (the basic gradient pattern) within the arterial wall could be maintained with the proper communication between ECs and VSMCs. Supporting this view, an even distribution of active VSMC in medial layer caused was found by EC-denudation. It is therefore conceivable that the gradient activation pattern of VSMCs could be necessary for integrity of the medial layer and thus an aberrant activation pattern of VSMCs could lead to various disease states including hypertension, cerebral/coronary vasospasms, and aortic aneurism.

Intimal denudation as well as L-NAME treatment failed to identify a significant difference of MLC phosphorylation from that of EC-intact aorta using a biochemical method (Fig. 1). On the other hand, the use of the imaging technique clearly demonstrated the notable difference in MLC phosphorylation within 2–3 layers of VSMCs near the intima (Fig. 3, A and B). The present results show a clear advantage of the use of the imaging technique over conventional biochemical approach to identify the phosphorylation level of proteins within the cells, such as MLC with spatiotemporal manners.

Another exciting finding of this study is that the phosphorylation level of MLC in the outermost medial layer was uniquely high in PE-stimulated SHR aorta in the presence of

ACh, whereas that was suppressed throughout entire smooth muscle layers in control WKY. This high MLC phosphorylation, thus activating smooth muscle cells unique to the outermost smooth muscle layers of SHR aorta is not simply attributed to NO diffusion considering the gradual change in pMLC in the absence of ACh (Fig. 6B).

There was no difference in the steady-state ACh sensitivity of aorta between SHR and WKY (Fig. 8A), suggesting that NO production of EC of SHR is similar to that of WKY. Therefore, high MLC phosphorylation of VSMCs at the outermost layer is likely to be due to the unique properties of VSMCs. Our data revealed that the activation level of VSMCs at the outermost layer in SHR during NO-induced relaxation is approximately 10 fold higher than that of WKY. This observation allowed us to predict that the unique characteristic of VSMCs in SHR could influence the widening of blood vessels and promote an increase in blood pressure. In favor of this notion is the recent report that increased aorta stiffness, a fundamental manifestation of hypertension, is attributable not only to change in the extracellular matrix (ECM), but also to correlate with the abnormal mechanical properties of VSMCs in SHR (Zhou et al., 2017). This study showed that the stiffness of single-VSMC measured by atomic force microscopy (AFM) was significantly increased in SHR compared with that in WKY. Interestingly, abnormal mechanical properties of VSMC observed in large arteries, but not in distal arteries. Taken together, these findings suggest that the unique characteristic of VSMCs in SHR may serve as one of the components associated with the development of hypertension.

In smooth muscle, the extent of MLC phosphorylation are controlled by the balance of MLCK and MLCP activity (Somlyo and Somlyo, 1994; Stull et al., 1991), thereby regulating the spatial and temporal activation of VSMCs in tissue. Therefore, it is possible that the expression and activation of these regulatory molecules in the outer layer of aorta in SHR are different from that of WKY. Smooth muscle MLCK has been reported to up-regulated in SHR in an age-dependent (blood pressures dependent) manner (Han et al., 2006). However, our results showed that the spatial patterns of VSMC activations and force developments in the PE-induced contraction were similar in both SHR and WKY, suggesting that agonist-stimulated contractile pathways including Ca^{2+} signaling and Ca^{2+} sensitization pathways are not significantly different in aorta tissues between SHR and WKY.

Since this difference between SHR and WKY is only found in the presence of ACh, it is plausible that the difference is attributed to the components of NO dependent cascade. It may be in part linked to the fact that the size of fenestrations in the internal elastic lamina are smaller in SHR than that of WKY (Sandow et al., 2009), suggesting that the rate of NO diffusion in the tunica media may be spatially restricted in SHR aorta. However, since the discretely high MLC phosphorylation was found at only the outermost medial layer of SHR and there is no evidence for a higher barrier for diffusion in only outer elastin layer in tunica media, it is unlikely that the difference in the fenestration explains the characteristic high MLC phosphorylation of SHR.

Since SHR is known as the genetic strain of hypertensive rat, it is important to investigate the unique characteristic of VSMC in other hypertension models such as endocrine hypertension, environmental hypertension, and pharmacological hypertension (Leong et al.,

2015; Sarikonda et al., 2009). Further studies are required to determine the components responsible for this unique change. However, it should be emphasized that our imaging approach enables us to detect the spatially unique properties of smooth muscle cell contractility within the tissue, which is critical to understand the mechanism of vascular diseases such as hypertension.

In the present study, we provide new insights into how differently individual VSMCs within distinct medial layers respond to ECs during the contraction-relaxation process under physiological conditions. Moreover, we addressed the question about how VSMCs in differential physical locations in the tissue change their function under pathological conditions, such as vascular injury and hypertension. Using a novel imaging approach, we show that individual smooth muscle cells have unique contractile activity in the tissue. The present study indicates that it is critical to analyze the activity of protein components important for the regulation and activation of smooth muscle cells at individual cell level in the tissues to understand the regulatory mechanism of contraction-relaxation processes of contractile tissues, including arteries but not excluding other tissues such as airways and gastrointestinal tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. VSMCs activation in aorta vessel segments

(A) Force traces of EC-intact and denuded rat thoracic aorta under various conditions. Averaged force traces with freezing noises in EC-intact (upper, I- indicates EC-intact aorta) and denuded rat aorta (lower, D- indicates EC-denuded aorta) were acquired at resting state (I-Rest, D-Rest), after addition of 10 μ M PE (I-PE, D-PE), PE in the presence of 1 μ M ACh (I-ACh, D-ACh), and PE in the presence of both 1 μ M ACh and 100 μ M L-NAME (I-LNAME). Relative force was normalized to a value of contraction stimulated with 124 mM KCl for 3 min. (B) Phosphorylation level of MLC in EC-intact (lanes 1–4) and denuded rat aorta (lanes 5 and 6) under various conditions. Amount of phosphorylated MLC was measured using Urea-glycerol gel electrophoresis (n=3; mean $\pm$ s.e.m)(see Methods for details). (C) Criterion for intactness and denudation of ECs in arteries. In all EC-intact aortic vessels used for this study, an administration of 1 μ M ACh reduced the PE-induced contraction to less than 25% (A/B in the figure) within 2 min.

Fig. 2. Spatiotemporal regulation of VSMCs in tunica media under influence of ECs in tunica intima

(A) Visualization of heterogeneous activation of VSMCs during PE-induced contraction in EC-intact rat thoracic aorta. At 60 sec after PE stimulation, EC-intact aorta were quick-frozen by plunging into liquid nitrogen-cooled propane and stained with pMLC and smMHC for the observation of the stimulus-specific activation of VSMCs in aorta tissues (see Methods for details). The multiple Z-series sections for each sample were projected onto one plane. pMLC, phosphorylated regulatory light chain of myosin; smMHC, smooth muscle myosin heavy chain; Sytox Green for nuclei. (B) Upper panel shows a pMLC/smMHC ratio image (SIRp). The quantified ratio intensity obtained by the longitudinal summation of SIRp was averaged (lower panel, $n=7$) and displayed along position from inner to outer layers of SMCs within tunica media. Bar, 50 μm . It should be noted that the lower panel shows the numerical value based on SIRp in the entire image of the upper panel b. Zero is taken arbitrary. (C) Numerical comparison of SIRp between inner (in) and outer (out) regions of tunica media in EC-intact aorta. The fluorescent signal intensities of SIRp of the image in B were longitudinally divided into inner and outer halves to quantitatively analyze the SIRp

values. The SIRp values were determined using NIH image program (mean $\pm$ s.e.m, n=7). The graph shows significant difference between inner and outer half region. a.u., arbitrary units.

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Fig. 3. Effect of ECs on the spatial activation of VSMCs in tunica media

(A) Effect of NO release from ECs on the spatial activation of VSMCs. Upper panels from left to right show ratio images at rest (I-Rest), PE in the presence of ACh (I-ACh), and PE in the presence of both ACh and L-NAME (I-LNAME) in the EC-intact vessels. Note that I- indicates EC-intact aorta. (B) Effect of the EC-denudation on the spatial activation of VSMCs. Upper panels from left to right represent resting (D-Rest), PE alone (D-PE), and PE in the presence of ACh (D-ACh) in the EC-denuded vessels. Note that D- indicates EC-denuded aorta. Lower panels in (A) and (B) show the quantified, averaged intensity of the ratio signals from inner to outer layers of VSMCs in tunica media ($n = 7$). Note that the ratio signals of I-PE and I-ACh are also shown as references. (C) Numerical comparison of SIRp between inner (in) and outer (out) regions of tunica media in EC-intact and denuded aorta under various conditions. The fluorescent signal intensities of SIRp of each image in Fig. 2B and Fig. 3A and B were longitudinally divided into inner and outer halves to quantitatively analyze the SIRp values. The SIRp values were determined using NIH image program (mean $\pm$ s.e.m, $n=7$). The graphs show significant difference between inner and outer half

region, Left graph, I-PE (in) vs. I-LNAME (in); Middle graph, I-PE (in) vs. D-PE (in); Right graph, I-ACh (in) vs. D-ACh (in), and I-ACh (out) vs. D-ACh (out). a.u., arbitrary units.

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Fig. 4. Different phosphorylation levels of MLC between inner and outer layers of VSMCs in response to ACh

(A) Phosphorylation level of MLC determined by using Urea-glycerol gel electrophoresis in inner and outer layers split from arterial rings in the presence and absence of ACh in PE-stimulated EC-intact and denuded vessels (see Methods for details). Upper, representative gel images; lower, statistic analysis. The values are mean $\pm$ s.e.m (n = 3). (B) Expression of various contractile and regulatory proteins influencing MLC phosphorylation in inner and outer layers from EC-denuded vessel. The samples were separated by 7.5–20% polyacrylamide gradient slab gel and followed by western blotting with various antibodies (smMHC, Rho-associated protein kinases (ROCK α and ROCK β), MLCK, myosin phosphatase target subunit 1 (MYPT1), protein kinase Gs (PKGI α and PKGI β), protein kinase C α (PKC α), α -actin, RhoA, and MLC).

Fig. 5. Effect of partial removal of ECs on VSMC activation during PE-induced contraction
 (A) Half-denuded aorta was stained with anti-pMLC Ab, anti-smMHC Ab and Sytox Green for nuclei after PE-stimulation. Double-head arrows indicate an area of the presence of ECs (small arrows in lower left panel show the position of EC nuclei). Bar, 50 μm. (B) Quantified ratio image of SIRp in the half-denuded aorta. (C) Relationship between the quantified ratio intensity summed vertically and location of half-denuded tunica media. Red dashed lines in (c) show the best-fit lines in the EC-denuded and intact regions (gray area). Note that the slope of the line from EC-intact region is steeper than that of denuded region, suggesting that the presence of EC decreases pMLC (-0.0703 ± 0.005 in EC-intact vs. -0.0267 ± 0.002 in EC-denuded, $p < 0.05$). The values are mean $\pm$ s.e.m ($n = 3$). (D) Averaged force traces of ACh-induced relaxation in PE-stimulated EC-intact, half-denuded, and denuded aorta. Arrow indicates an addition of 1 μM ACh.

Fig. 6. Distinct spatial patterns of VSMC activation between spontaneously hypertensive rat (SHR) and normotensive Wister-Kyoto rat (WKY)

(A) Intensity distribution of the ratio in intact WKY (WKY-PE) and SHR (SHR-PE) aortas after PE-stimulation. Bar, 50 μ m. (B) Relationship between the ratio intensity (summed from the top to the bottom in A; mean $\pm$ s.e.m, n=7) and position within tunica media of WKY (red) and SHR (black). (C) Force traces of PE-induced contraction in SHR and WKY aortas. (D) Differential activation of VSMCs in SHR (SHR-ACh) compared to WKY (WKY-ACh) after PE stimulation in the presence of ACh. (E) Quantified ratio signals in SHR and WKY in the presence of ACh (n=7). (F) Force traces of PE-induced contraction in the presence of ACh in SHR and WKY.

Fig. 7. Phosphorylation levels of MLC in inner and outer layers in EC-intact WKY and SHR aortas

(A)(B) Rat aorta rings were treated with 10 μ M PE with or without ACh and subjected to Urea-glycerol gel electrophoresis followed by immunoblotting. Upper, representative gel staining; lower, statistic analysis of upper trace. Distinct amount of pMLC in the inner and outer layers in both WKY and SHR after PE stimulation was detected with the similar pattern as seen in Fig 6A (see Methods for details). The values are mean $\pm$ s.e.m (n = 3). Although a band of pMLC in the outer layer of SHR in the presence of ACh was detected, that of WKY is only modestly detected.

Fig. 8. Comparisons of aorta tissues from spontaneously hypertensive rat (SHR) and normotensive Wister-Kyoto rat (WKY)

(A) ACh sensitivity of aortas from Wister, WKY, and SHR rats. Sensitivity to ACh in 10 μM PE-induced contraction was not significantly different between ~9 weeks old (according to a chart from the Charles River Lab.) Wister, 12 weeks old WKY, and 12 weeks old SHR rats.

(B) Elevated arterial wall thickness in SHR. Wall thickness of aorta tunica media was measured from DIC images after formaldehyde fixation in WKY ($86.37 \pm 1.920 \mu\text{m}$, n=25) and SHR ($93.51 \pm 1.366 \mu\text{m}$, n=41).