Enhanced contraction and myosin phosphorylation induced by Ca^{2+}-independent MLCK in spontaneously hypertensive rats

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Directed by Professor Young-Ho Lee

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Young-Eun Cho

June 2010

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June 2010

Acknowledgements

I am really thankful to God for giving me a chance to study here. I believe that You prepare a good plan for me as You have done until now.

Professor Young-Ho Lee, I appreciate your deep concern for 7 years. I am very proud to be your disciple. Professor Duck Sun Ahn, thank you for the advice, which gave me much to think about. Professor Taick Sang Nam, Yang-Soo Jang and Ki-Chul Hwang, thank you for your comments and advice for my work. Professor Ji-Soo Yoo, Sang Hui Chu and Hyangkyu Lee, your advice and guidance were really great. Thank you so much.

Young-Hwan and Soo-Kyoung, you have inspired and supported me. Ph.D. Eok-Cheon Kim and MiHwa, even though we could not spend much time, you were so good to me. Shin-Woo Kang and Jin-Hye Choi, you were really good supporters and helpers. Ji-Young, Hyuna, Moon-Chul and YongJoon, I will keep all our memories in my mind. Hyung Shin and Jin-Young, thanks for your kind cares. Thanks to all people of department of physiology, wholeheartedly.

My friends, JiNa, JiYoung, SunJeong, SunHa and Hong, I love you all guys. Thank you for being with me. Eunice and Mark, I think we are friends now, wish you become great. My kids in JCM, you refreshed me with your pure hearts every Sunday. Teachers who prayed for me, I remember all, thank you.

Finally, my mother, father and uncle, Prof. Kwon, this would have been impossible without you. I love you and respect you from the bottom of my heart. Please, take care and be strong in God.

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Abstract

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The aim of this study was to clarify the role of a possible Ca^{2+}-independent myosin light chain kinase (MLCK) activity in hypertension. The increase in contractile force and phosphorylation of 20kDa regulatory light chains of myosin II (MLC_{20}) evoked by the type 1 and 2A phosphatase inhibitor, calyculin A in β-escin permeabilized mesenteric arteries at pCa9.0 were compared between Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). There is no detectable phosphorylation of MLC_{20} in permeabilized arteries at pCa9.0, but the administration of 1μM calyculin A gradually increased force and mono- and diphosphorylation of MLC_{20}. This contraction is inhibited by staurosporine, a broad-spectrum protein kinase inhibitor but not by wortmannin, Y-27632, or calphostin-C. The calyculin A-induced contraction is significantly greater in SHR than WKY and it was associated with an increase in mono- and diphosphorylation of MLC_{20}. SM-1, a ZIPK inhibiting peptide, significantly inhibits the amplitude of the calyculin A-induced contraction. Total ZIPK expression (54 and 32 kDa) is greater in SHR than WKY. Phosphorylation

of MYPT1 at Thr-697, but not at Thr-855, is consistently stronger in SHR compared with WKY in calyculin A-treated tissues at pCa9.0. These results suggest that a Ca^{2+}-independent MLCK activity is enhanced in SHR and ZIPK is the key candidate for this kinase in rat mesenteric arteries. Furthermore, the higher expression level of ZIPK in SHR appears to increase Thr-697 phosphorylation of MYPT1, MLC_{20} phosphorylation and contraction while calyculin A induces phosphatase inhibition in SHR.

Keywords : Ca^{2+}-independent MLCK, ZIPK, hypertension, MLC_{20} diphosphorylation

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Ⅰ. *Introduction*

Smooth muscle contraction is elicited primarily by phosphorylation at Ser-19 of the 20kDa regulatory light chains of myosin II (MLC_{20}), which is controlled by the opposing activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). Therefore, the amplitude of contraction can be affected by any changes in the ratio of MLCK:MLCP activity. MLCK activity is dependent on Ca^{2+}-calmodulin, hence, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the primary determinant of smooth muscle contraction[1-3]. However, both contractile force development and MLC_{20} phosphorylation in smooth muscle can be also elicited without corresponding changes in $[Ca^{2+}]_i$. The increase in the sensitivity of smooth muscle contractile response to $[Ca^{2+}]_i$ (i.e. Ca^{2+} sensitization) can be caused by the inhibition of MLCP activity or activation of extracellular regulated kinase (ERK)-mediated caldesmon phosphorylation[4]. MLCP, type 1 protein serine/threonine phosphatase, consists of catalytic subunit (PP1c), myosin phosphatase targeting subunit (MYPT1) and unknown subunit (M_{20})[2]. It can be inhibited either directly by phosphorylation of MYPT1 or indirectly via phosphorylation of protein kinase C (PKC)-potentiated phosphatase inhibitor protein of 17kDa (CPI-17)[5]. Thr-697 and

Thr-855 of MYPT1 (rat sequence) have been identified as key inhibitory phosphorylation sites for the regulation of MLCP activity. These sites can be phosphorylated by RhoA-associated kinase (ROCK)[6,7], zipper-interacting protein kinase (ZIPK)[8,9] and integrin-linked kinase (ILK)[10,11]. ROCK[12], ZIPK[13] and ILK[14] can also phosphorylate CPI-17. CPI-17 is activated by Thr-38 phosphorylation, which inhibits PP1c activity in vascular smooth muscle[15-17]

Another possible mechanism for Ca^{2+} sensitization also exists. It has been elucidated by the protein phosphatase inhibitor-induced contraction in the absence of Ca^{2+}. When type 1 and 2A phosphatase inhibitor, microcystin-LR is applied to β-escin permeabilized rat caudal arterial strips at pCa9.0, sustained contraction is observed that cannot be attributed to traditional MLCK activity[9,18,19]. It is accompanied with the increase of mono- (Ser-19) and di- (Ser-19/Thr-18) phosphorylation of MLC_{20}. Similar results have been obtained with various types of smooth muscle tissue and other inhibitors of type 1 and 2A phosphatase, such as okadaic acid and calyculin A[18,20-22], suggesting that is a common mechanism. Microcystin-LR-induced contraction at pCa9.0 was inhibited by staurosporine, broad-spectrum protein kinase inhibitor but was unaffected by several other protein kinase inhibitors, including ROCK inhibitor Y-27632, PKC inhibitor GF-109203X, MLCK inhibitor ML-7, and MLCK and phosphatidylinositol 3-kinase inhibitor wortmannin. These results suggested that Ca^{2+}-independent MLCK may exist in vascular smooth muscle[9,19]. Since traditional MLCK is absolutely dependent on Ca^{2+} at physiological levels of the kinase and specific for Ser-19[23], Ca^{2+}-independent MLCK may be not MLCK itself clearly. Many previous reports have suggested several candidates for Ca^{2+}-independent MLCK. Among them, zipper-interacting protein kinase (ZIPK) and integrin-linked kinase (ILK) can phosphorylate MLC_{20} at both Ser-19 and Thr-18 in the absence of intracellular Ca^{2+}. Therefore, ZIPK[9,24-26] and ILK[27,28] have emerged as candidates of Ca^{2+}-independent MLCK.

MLC_{20} monophosphorylation is most commonly observed in smooth muscle tissues in response to physiological contractile stimuli and low levels (<10%) of MLC_{20} diphosphorylation have been reported in a variety of vascular smooth muscles[29,30]. However, earlier reports showed that diphosphorylated MLC_{20} has a higher actin-activated Mg^{2+}-ATPase activity than monophosphorylated MLC_{20}[23,31,32]

and diphosphorylated MLC_{20} enhances the tension acting on stress fibers in fibroblasts[33]. Additionally, in vasospasm models, diphosphorylated MLC_{20} was significantly increased and correlated with a decreased diameter of cerebral arteries[34,35] and coronary arteries[36]. These results have led to the suggestion that diphosphorylation of MLC_{20} is associated with the hyper-contractility in vascular smooth muscle. Hypertension is also a vascular disease accompanied by hyper-contractility in vessels with many unknown reasons[37]. However, the relationship between diphosphorylated MLC_{20} and hyper-contractility of hypertension has not been elucidated.

Therefore, in this study, it was investigated whether the contractile force and MLC_{20} diphosphorylation evoked by Ca^{2+}-independent MLCK increase in the hypertensive model. Furthermore, to determine the mechanisms concerned with the augmentation of contractile force and MLC_{20} diphosphorylation induced by Ca^{2+}-independent MLCK in hypertension, phosphorylation of MYPT1 and CPI-17 was investigated.

//. *Materials and methods*

This investigation confirms with the Guide *for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

1. Tissue preparation and organ bath study

Male WKY and SHR aged 16~18 weeks were purchased from Japan SLC, Inc Inasa Production Facility. The average systolic blood pressures of WKY and SHR were 145±10.5mmHg and 202±10mmHg, respectively. Rats were killed by a decapitation, according to protocols approved by the Institutional Animal Care and Use committee.

All mesenteric arteries were excised and placed in HEPES-Tyrode solution (mmol/L : 10 Glucose, 10 HEPES, 134 NaCl, 5.6 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$) which was aerated by 100% O_2. The 2^{nd} or 3^{rd} branches of mesenteric arteries were prepared as ring segments (about 1mm length). Fat and adventitia were removed mechanically under binocular microscope and endothelium of the rings was removed by gentle rubbing the endothelium surface. Force was measured as described previously[38]. Briefly, each arterial ring was mounted onto an isometric transducer in a temperature-controlled 3ml organ bath horizontally (UFER, Medical Kishimoto, Kyoto, Japan). After an equilibration for 30min in HEPES-Tyrode solution aerated by 100% O_2 continuously, the ring segments were stretched passively by imposing an optimal resting tension, 5mN, which was evaluated as a maximum force developed by 70 mmol/L K^+ HEPES-Tyrode solution (K^+ substitution for Na^+). Arterial rings were repeatedly contracted with 70 mmol/L K^+ HEPES-Tyrode solution until stable responses were obtained.

2. β-escin permeabilization and force recording

Force in permeabilized preparation was measured as described previously[39]. Briefly, after obtaining the stable contractile response with 70 mmol/L K^+ HEPES-Tyrode solution from each vessel segment, arterial rings were permeabilized with

50μM β-escin for 30min in a relaxing solution (mmol/L : 74.1 potassium methanesulfonate, 2 magnesium methanesulfonate, 4.5 MgATP, 1 EGTA, 10 creatine phosphate, 30 PIPES, pH 7.4) at 24℃. In pCa solution, 10mM EGTA was used to give a desired concentration for free Ca^{2+}. Ionic strength was constant at 0.2M by adjusting the concentration of potassium methanesulfonate. After permeabilization, vessels were contracted with pCa4.5 solution and relaxed upon return to pCa9.0. 1μM calyculin A and several protein kinase inhibitors were treated after stable responses were obtained at pCa9.0. All experiments, except the study with protein kinase inhibitors and inhibiting peptides, were conducted in 10μM wortmannin-pretreated tissue to eliminate the possible contribution of Ca^{2+}-dependent MLCK.

3. MLC_{20} phosphorylation measurement

To compare of mono- and diphosphorylated MLC_{20} between WKY and SHR, urea-glycerol minigel electrophoresis was used. When contractile responses became stable at pCa9.0, pCa4.5 and pCa9.0 with calyculin A, vessels were quickly frozen by immersion in dry-ice/acetone slurry containing 10% trichloroacetic acid (TCA) and 10mM dithiothreitol (DTT). After incubating in dry-ice/acetone slurry for 4hrs at 4℃, vessels were washed with 10mM DTT in acetone to remove TCA and then air-dried for 1hr. All segments were kept in -70℃ until use. Protein was extracted with a urea sample buffer (20mmol/L Tris, 23mmol/L glycine, 8mol/L urea, 10% glycerol, 10mmol/L DTT and 0.04% bromophenol blue). After vortexing every 15mins for 2hrs at room temperature, supernatant was loaded to 12.5% glycerol-urea gels (40% glycerol, 7.5% acrylamide, 0.32% bisacrylamide, 20mmol/L Tris, and 23mmol/L glycine) in running buffer (mmol/L : 20 Tris base, 23 glycine, 1 DTT and 1 sodium thioglycolate) with 300V constant voltage for about 5hrs. It transferred to nitrocellulose membrane at 100V for 100mins in transfer buffer (247mmol/L Tris, 192mmol/L glycine and 20% methanol). The membrane was incubated for 1hr at room temperature with 5% nonfat dry milk in PBS (mmol/L : 137 NaCl, 2.7 KCl, 10.14 Na_2HPO_4, 1.76 KH_2PO_4) with 0.05% Tween-20. It was incubated with total MLC_{20} antibody (1:500, Sigma, St. Louis, MO, USA) for 2hrs at room temperature. Anti-mouse IgM (goat) conjugated with horseradish peroxidase, was used as secondary antibody (1:2000; Calbiochem, Darmstadt, Germany). Blots were

developed with enhanced chemiluminescence (Santa Cruz Biotechnology, Inc., CA, USA) and then the bands were quantified by densitometry (Fuji film Science lab 2005, Image Gauge Version 3.0).

4. SDS-PAGE and western blotting

The expression level of proteins in non-stretched mesenteric arteries and the phosphorylation level of MLC_{20}, MYPT1 and CPI-17 in β-escin permeabilized mesenteric arteries were measured by SDS-PAGE and western blotting. Non-stretched arteries and permeabilized arteries after functional study were frozen by immersing in dry-ice/acetone slurry with 10% trichloroacetic acid (TCA) and 10mM dithiothreitol (DTT). They were washed with 10mM DTT in acetone to remove TCA and then air-dried for 1hr. All segments were kept in -70℃ until use. Vessels were homogenized with lysis buffer (sucrose 0.32mol/L, EDTA 2mmol/L, SDS 1%) and centrifuged at 13000g for 10min 4℃. Homogenates were resolved on 12 % SDS-PAGE gels (12% acrylamide, 0.32% bisacrylamide, 0.37mol/L Tris[pH 8.8], and 0.1% SDS) in SDS-PAGE running buffer (247mmol/L Tris, 192mmol/L glycine and 1% SDS). It transferred to nitrocellulose membrane at 100V for 100min in transfer buffer. The membrane was incubated for 1hr at room temperature with 5% nonfat dry milk or 5% BSA (only for phosphorylated proteins) in PBS with 0.05% Tween-20. It was incubated with primary antibodies; phospho-MLC_{20} (1:500, Cell Signaling, MA, USA), phospho-MYPT1 (1:500, Upstate, MA, USA), total MYPT1 (1:500, BD Transduction Laboratories™, CA, USA), phoshpo-CPI-17 (1:200, Santa Cruz Biotechnology, Inc., CA, USA), total CPI-17 (1:250, abcam, Cambridge, UK), ROCK (1:250, Upstate, MA, USA), ILK (1:250, Santa Cruz Biotechnology, Inc., CA, USA) and ZIPK (1:250, Calbiochem, NJ, USA) for 2hrs at room temperature or overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1hr at room temperature. Blots were developed with enhanced chemiluminescence and then the bands were quantified by densitometry.

5. Immunofluorescence staining

To examine the protein expression levels, the 1st or 2nd branches of mesenteric artery were dissected and then fixed in 4% paraformaldehyde (PFA) for 1hr at room temperature. To compare of MLC_{20} diphosphorylation level in calyculin A-induced contraction between WKY and SHR, arteries were fixed in the organ chamber after functional study and incubated with 4% PFA for 1hr at room temperature. Fixed arterial segments were embedded in optimal cutting temperature compound (OCT, Sakura Fine Tec Inc., Torrance, CA, USA) and frozen in the liquid nitrogen and kept in -70℃ until use. Arteries were sectioned into 10μm thick slices and mounted on gelatin-coated slides. After air-dry for 1hr, slides were washed twice for 10mins with PBS. After blockade for 4hrs in PBS containing 3% BSA and 0.5% Triton X-100, sections were incubated with primary antibodies for overnight in humidified chambers at room temperature. Antibodies were diluted in blocking solution; ZIPK (Santa Cruz Biotechnology, Inc., CA, USA) 1:50, total MLC_{20} (Sigma, St. Louis, MO, USA) 1:100, β-actin (abcam, Cambridge, UK) 1:500 and phospho-MLC_{20} Ser-19/Thr-18 (Cell Signaling, MA, USA) 1:100. After washing 6 times for 30mins with PBS, sections were incubated with secondary antibodies for 1hr in dark (1:500, Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 594 anti-goat IgG, CA, USA). After washing, sections were mounted with a mounting medium and sealed under a cover slip. Immunofluorescence signals were observed with confocal microscope (Carl Zeiss, LSM 510).

6. Drugs

Calyculin A, wortmannin and Y-27632 (trans-4-[(1R)-1-Aminoethyl]-N-4-pyridinylcy-clohexanecarboxamide) were purchased from Enzo Life Science (Farmingdale, NY, USA). Calphostine-C and staurosporine were purchased from Sigma (St. Louis, MO, USA). ZIPK inhibiting peptide, SM-1 (sequence:AKKLSKDRMKKYMARRKWQKTG) and scrambled SM-1 peptide (sequence:KWARMKDKAMRYKTKGRLQKSK) were purchased from BIO BASIC INC. and Thermo SCIENTIFIC. General laboratory reagents were of analytical grade or better.

7. Statistics

Data are expressed as mean±SE. The magnitude of contraction was expressed as a relative percentage of the contraction at pCa4.5. Differences between means were tested by ANOVA following Tukey's test. Significant differences were taken at the $p<0.05$ level.

///. *Results*

1. Characterization of Ca^{2+}-independent and calyculin A-induced contraction in β-escin permeabilized rat mesenteric artery

Figure 1A depicts a typical calyculin A-induced contraction of β-escin permeabilized rat mesenteric arterial smooth muscle in the absence of Ca^{2+}. After Ca^{2+}-induced contraction was confirmed with pCa4.5 solution, calyculin A was treated at pCa9.0. As shown in Figure 1A, the administration of 1μM calyculin A to the permeabilized rat mesenteric artery at pCa9.0 elicited a gradual increase in force, reaching a plateau 20~30min after administration. The magnitude of contraction was about 66.23±5.35% (n=15), which is a relative contraction to maximum of pCa4.5-induced contraction. To clarify which kinase is responsible for the calyculin A-induced Ca^{2+}-independent contraction, it was examined the effect of various protein kinase inhibitors on calyculin-A induced contraction at pCa9.0 (Fig.1B-E). Pretreatment of MLCK inhibitor wortmannin (10^{-5}M, n=12), ROCK inhibitor Y-27632 (10^{-5}M, n=4) and PKC inhibitor calphostin C (5×10^{-7}M, n=7) had no effect on the calyculin A-induced contraction at pCa9.0. However, non-specific kinase inhibitor, staurosporin almost completely inhibited calyculin A-induced contraction at pCa9.0 (10^{-5}M, n=4). Figure 1F shows the statistic results of the effect of various inhibitors on calyculin A-induced contraction.

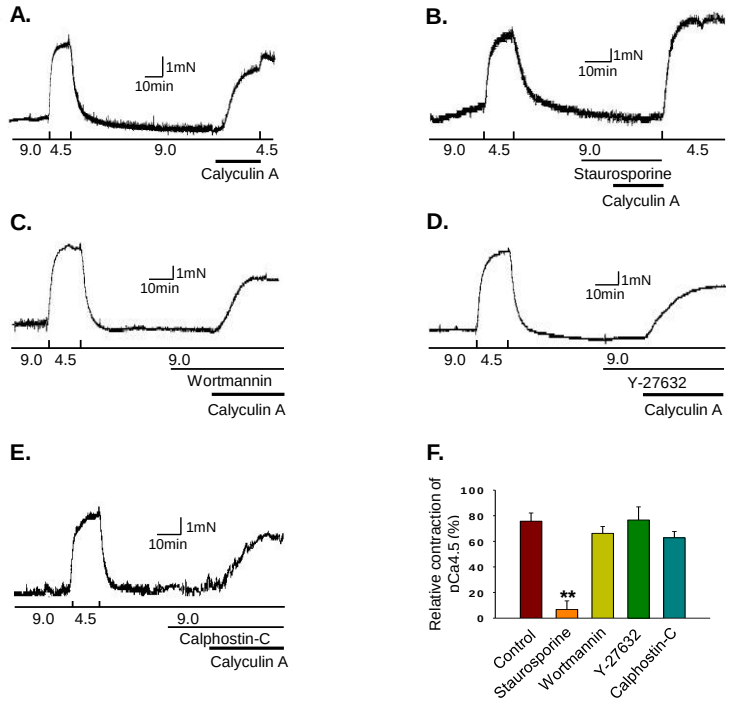

Figure 1. Ca^{2+}-independent and calyculin A-induced contraction in β-escin permeabilized rat mesenteric arterial smooth muscle. A. Typical record of calyculin A-induced contraction in β-escin permeabilized rat mesenteric artery at pCa9.0. B-E. Typical record of calyculin A-induced contraction at pCa9.0 with protein kinase inhibitors; 10^{-5}M staurosporine (B), 10^{-5}M wortmannin (C), 10^{-5}M Y-27632 (D) and 5×10^{-7}M calphostin-C (E) were applied 15 min before calyculin A treatment. F. Mean data of calyculin A-induced contraction with protein kinase inhibitors at pCa9.0. Results are expressed as mean±SE (n=4~9). The magnitude of contraction was normalized by the response of pCa4.5. Asterisk indicates $p<0.01$ (**) compared with the value of control.

2. Comparison of Ca^{2+}-independent and calyculin A-induced contraction and MLC_{20} phosphorylation between WKY and SHR

To clarify whether the Ca^{2+}-independent and calyculin A-induced contraction is increased in a hypertensive model, calyculin A-induced contraction in β-escin permeabilized rat mesenteric arterial smooth muscle of WKY and SHR was compared. As shown in Figure 2A, calyculin A-induced contraction was concentration-dependent. The maximal contraction was obtained at 1μM in both WKY and SHR, but the amplitude of calyculin A-induced contraction was greater in SHR than WKY at all concentrations except 10^{-8}M ($n=4$, $p<0.05$).

To confirm the correlation between changes in calyculin A-induced contraction and MLC_{20} phosphorylation in the hypertensive model, MLC_{20} phosphorylation levels were compared between WKY and SHR in three different conditions using urea-glycerol minigel electrophoresis with total MLC_{20} antibody. As shown in Figure 2B, in β-escin permeabilized arteries, phosphorylated MLC_{20} was not observed at pCa9.0 in both WKY and SHR. At pCa4.5, un- and monophosphorylated MLC_{20} were observed in both WKY and SHR and the monophosphorylated MLC_{20} was greater in SHR compared to WKY. Diphosphorylated MLC_{20} was not detected at pCa4.5. However, calyculin A treatment at pCa9.0 induced three-bands, un-, mono- and diphosphorylated MLC_{20} in both WKY and SHR. Both mono- and diphosphorylated MLC_{20} were greater in SHR compared to WKY.

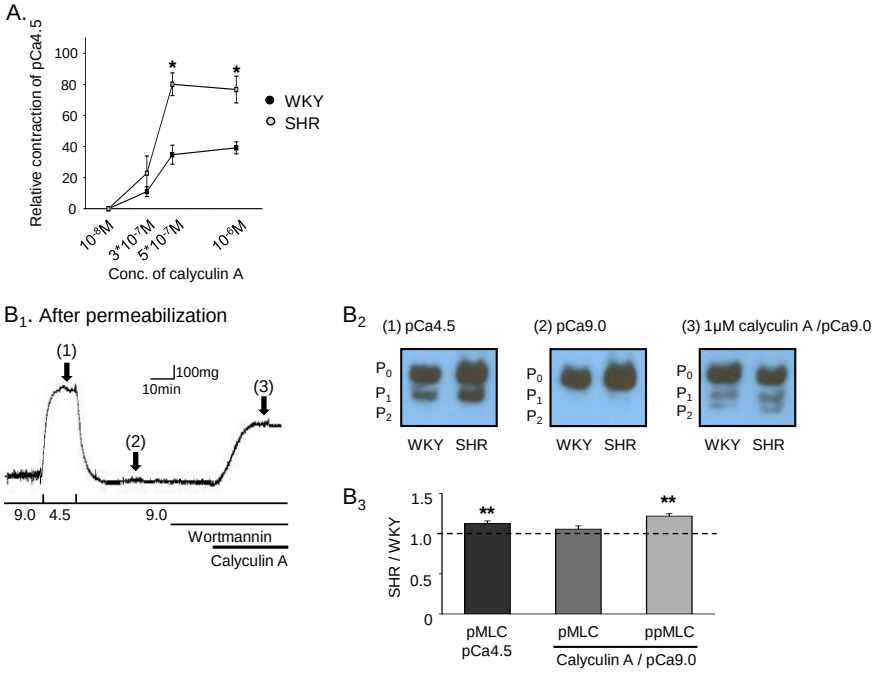

Figure 2. Changes in Ca^{2+}-independent and calyculin A-induced contraction and MLC_{20} phosphorylation in WKY and SHR. A. Concentration-dependent contraction induced by calyculin A in β-escin permeabilized rat mesenteric artery of WKY and SHR at pCa9.0. The magnitude of contraction was normalized by the response of pCa4.5. Asterisk indicates $p<0.05$(*) for the difference between WKY and SHR (n=4). B_1-B_3. MLC_{20} phosphorylation in three different conditions after β-escin permeabilization. Vessels were collected at the peak contraction induced by pCa4.5 (1), at pCa9.0 (2) and at the peak contraction induced by calyculin A at pCa9.0 (3). Unphosphorylated MLC_{20} (P_0), monophosphorylated MLC_{20} (P_1) and diphosphorylated MLC_{20} (P_2) were separated by urea-glycerol minigel electrophoresis. The densitometric sum of the bands was normalized to the value of WKY in each category (B_3). Asterisk indicates $p<0.01$(**) for the difference between WKY and SHR (n=4~5).

The level of mono- and diphosphorylated MLC_{20} induced by calyculin A was also compared using SDS-PAGE with specific antibodies for Ser-19 and Ser-19/Thr-18 phosphorylated MLC_{20}. As shown in Figure 3, the results are similar to those observed in urea-glycerol minigel electrophoresis. In WKY and SHR, Ser-19 phosphorylated MLC_{20} was increased at pCa4.5 and at pCa9.0 with calyculin A (Fig. 3A). Ser-19/Thr-18 diphosphorylated MLC_{20} was significantly increased at pCa9.0 with calyculin A (Fig. 3B). Quantification of the immunoblots showed that Ser-19 phosphorylated MLC_{20} at pCa4.5 and at pCa9.0 with calyculin A is stronger in SHR than WKY. However, diphosphorylated MLC_{20} was stronger in SHR than WKY only at pCa9.0 with calyculin A.

Finally, increased diphosphorylated MLC_{20} induced by calyculin A in the absence of Ca^{2+} was confirmed with immunofluorescence method. As shown in Figure 3C, diphosphorylated MLC_{20} was not observed at pCa9.0, whereas it was significantly increased with calyculin A treatment in both WKY and SHR. The fluorescence signal from diphosphorylated MLC_{20} induced by calyculin A was stronger in SHR than WKY. These results suggest that calyculin A-induced contraction at pCa9.0 was accompanied by the increase in mono- and diphosphorylation of MLC_{20}.

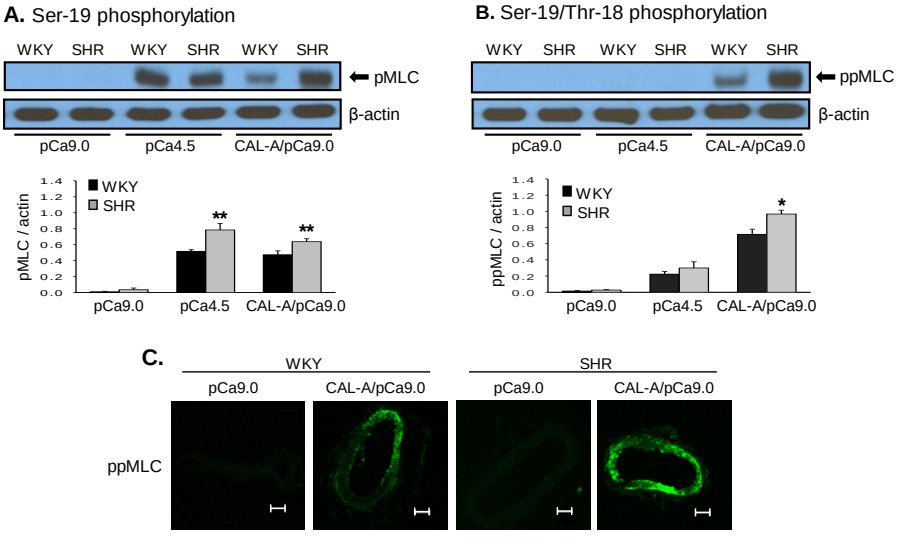

Figure 3. Calyculin A-induced Ser-19 and Ser-19/Thr-18 phosphorylation of MLC_{20} in WKY and SHR. A & B. Ser-19 phosphorylated MLC_{20} (A) and Ser-19/Thr-18 phosphorylated MLC_{20} (B) in WKY and SHR at pCa9.0, pCa4.5 and pCa9.0 with calyculin A. It was measured by SDS-PAGE with specific antibodies for Ser-19 and Ser-19/Thr-18 phosphorylation of MLC_{20}. Phosphorylation levels were normalized with β-actin. Results are expressed as mean±SE. Asterisks indicate $p<0.01$ (**) and $p<0.05$ (*) for the difference between WKY and SHR in each condition (n=4~9). C. Immunofluorescence staining for Ser-19/Thr-18 diphosphorylated MLC_{20} at pCa9.0 and pCa9.0 with calyculin A in WKY and SHR. Data were obtained from confocal microscopy. Scale bar : 50μm. pMLC : Ser-19 phosphorylated MLC_{20}, ppMLC : Ser-19/Thr-18 phosphorylated MLC_{20}. CAL-A : calyculin A

3. Identification of Ca^{2+}-independent MLCK in rat mesenteric artery

To identify the nature of Ca^{2+}-independent MLCK, it was investigated that the effect of SM-1, ZIPK inhibiting peptide, on the calyculin A-induced contraction in β-escin permeabilized rat mesenteric artery. As shown in Figure 4A, pre-treatment of SM-1 (500μM) significantly inhibited calyculin A-induced contraction, but scrambled SM-1 peptide had no effect on calyculin A-induced contraction. The expression of ZIPK was clarified by double immunofluorescence staining for ZIPK and β-actin or MLC_{20} in the cross-section of rat mesenteric arteries. Fluorescence signal of ZIPK was localized in the cytoplasm of the smooth muscle as verified with smooth muscle specific actin or myosin light chain. The negative control obtained without primary antibodies showed no detectable signal.

To determine whether expression of ZIPK is changed in SHR, the expression of ZIPK in rat mesenteric artery was compared between WKY and SHR using western blot. Expression levels of ROCK, CPI-17, and ILK were also compared. As shown in Figure 4C, anti-ZIPK antibody detected a prominent both band of 54 kDa and 32 kDa in SHR and weaker bands in protein-matched WKY tissues. Quantification of the immunoblots showed that total ZIPK (54 and 32 kDa) is 1.30 ± 0.04-fold (n=23) more abundant in SHR than in protein-matched samples from WKY. ROCK (180 kDa) was detected in both WKY and SHR tissues and the signal was consistently stronger in SHR compared with WKY (1.38 ± 0.06-fold, n=12). However, the expression levels of CPI-17 (0.89±0.05-fold, n=7) and ILK (0.94±0.05-fold, n=7) were not significantly different between WKY and SHR.

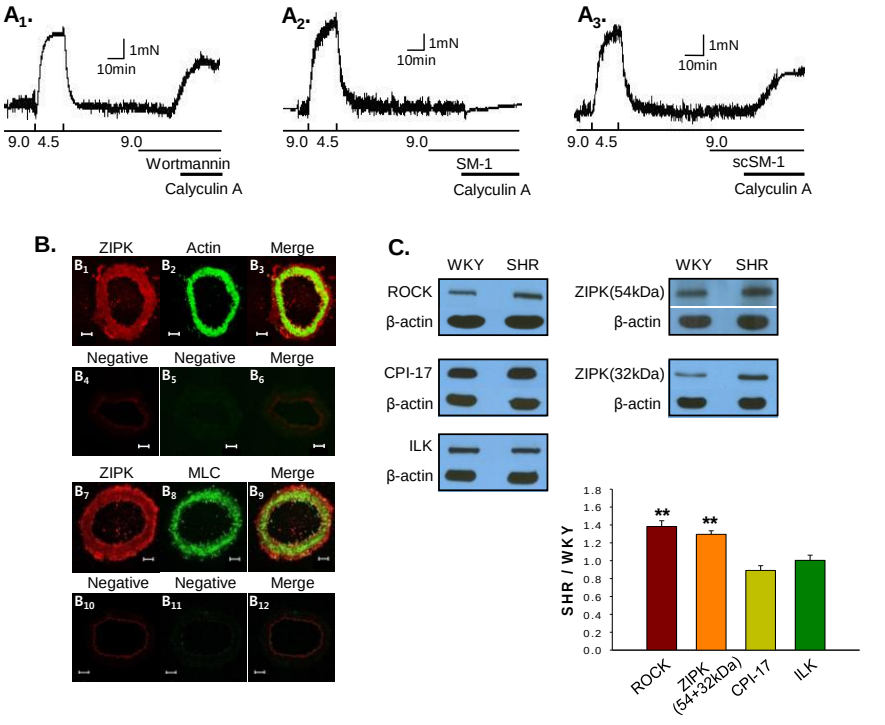

Figure 4. Effect of SM-1 on Ca^{2+}-independent and calyculin A-induced contraction and expression of various protein kinases in WKY and SHR. A_1-A_3. Effect of ZIPK inhibiting peptide, SM-1 on Ca^{2+}-independent and calyculin A-induced contraction in β-escin permeabilized rat mesenteric artery. Calyculin A was applied in the absence of SM-1 (A_1), in the presence of 500μM SM-1(A_2) and in the presence of 500μM scrambled SM-1(A_3). B. Double immunofluorescence staining for ZIPK and β-actin or MLC_{20} in the cross-section of rat mesenteric artery. Representative confocal images indicate ZIPK (B_1,B_7), β-actin (B_2) and total MLC_{20} (B_8). Negative controls were produced by omitting the primary antibodies ($B_{4\text{-}5}$, $B_{10\text{-}11}$). C. Immunoblots of ROCK, ZIPK, CPI-17 and ILK in the mesenteric artery of WKY and SHR. Protein expression level was normalized to the value of WKY in each category. Results are expressed as mean±SE. Asterisks indicate $p<0.01$ (**) for the difference between WKY and SHR.

4. Contribution of MYPT1 and CPI-17 phosphorylation during Ca^{2+}-independent and calyculin A-induced contraction in WKY and SHR

To determine the mechanism of calyculin A-induced increase of contraction and MLC_{20} phosphorylation in SHR, the difference in MYPT1 and CPI-17 phosphorylation by calyculin A was compared between WKY and SHR. As shown in Figure 5A, phosphorylation of MYPT1 at Thr-697 was significantly increased by calyculin A, which was greater in SHR than WKY (1.68±0.3 vs 1.06±0.14, fold of phospho-MYPT1 to total MYPT1, n=6). Phosphorylation of MYPT1 at Thr-855 was also significantly increased by calyculin A, however, it was similar between WKY and SHR (1.74±0.35 vs 1.70±0.36, fold of phospho-MYPT1 to total MYPT1, n=5). Phosphorylation of CPI-17 at Thr-38 was also significantly increased by calyculin A, however, it was similar between SHR and WKY (0.38±0.09 vs 0.34±0.07, fold of phospho-CPI-17 to total CPI-17, n=5).

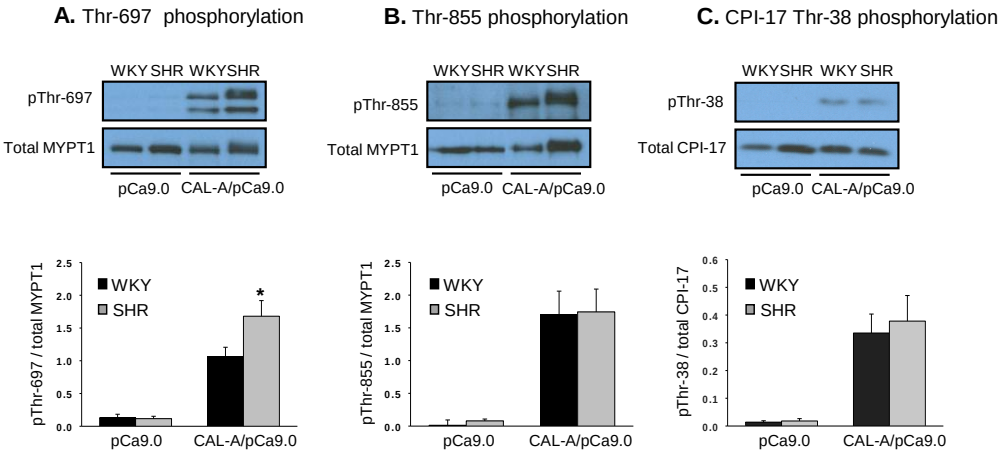

Figure 5. MYPT1 and CPI-17 phosphorylation during Ca^{2+}-independent and calyculin A-induced contraction in WKY and SHR. MYPT1 Thr-697 (A) and Thr-855 (B) phosphorylation and CPI-17 Thr-38 (C) phosphorylation at pCa9.0 and pCa9.0 with calyculin A in β-escin permeabilized mesenteric artery in WKY and SHR. Phosphorylation level was normalized with total MYPT1 (A,B) and total CPI-17 (C), which was compared between WKY and SHR. Results are expressed as mean±SE. Asterisks indicate $p<0.05$ (*) compared WKY and SHR. CAL-A : calyculin A

Ⅳ. Discussion

The present study demonstrates that Ca^{2+}-independent and calyculin A-induced contraction was significantly greater in SHR than WKY and the augmentation of contraction in SHR was concerned with the increase of mono- and diphosphorylation of MLC_{20}. It also shows that ZIPK may be the Ca^{2+}-independent MLCK involved in Ca^{2+}-independent and calyculin A-induced contraction, because SM-1 (ZIPK inhibiting peptide) significantly inhibited the amplitude of contraction induced by calyculin A at pCa9.0. The fluorescence signal of ZIPK was observed in the cytoplasm of rat mesenteric arterial smooth muscle and the expression level of ZIPK was more abundant in SHR than WKY. The increase of Thr-697 phosphorylation of MYPT1 by calyculin A in SHR was accompanied with the enhanced calyculin A-induced contraction and MLC_{20} phosphorylation in SHR. Taken together, these results suggest that Ca^{2+}-independent MLCK, ZIPK, plays an important role in the hypercontractility of vascular smooth muscle in SHR and possibly in other models of hypertension.

To investigate the existence of Ca^{2+}-independent MLCK and diphosphorylation of MLC_{20} in vascular smooth muscle, phosphatase 1 and 2A inhibitor, calyculin A was treated to β-escin permeabilized rat mesenteric artery at pCa9.0. Calyculin A evoked contraction and mono- and diphosphorylation of MLC_{20} at pCa9.0. Traditional MLCK activity is absolutely dependent on Ca^{2+} and calmodulin. Thus, under this experimental condition (pCa9.0), no phosphorylation of MLC_{20} was observed (Fig. 2). Inhibitors of MLCK, ROCK and PKC did not affect to calyculin A-induced contraction. However, it was inhibited by non-specific kinase inhibitor, staurosporine. These results are consistent with other previous studies[9,19]. It suggests that Ca^{2+}-independent MLCK contributes to the generation of contractile force in rat mesenteric artery and endogenous Ca^{2+}-independent MLC kinase activity is unmasked in this experimental condition.

To clarify whether Ca^{2+}-independent MLCK contributes to hypertension, calyculin A-induced contraction and MLC_{20} phosphorylation in β-escin permeabilized mesenteric artery at pCa9.0 were compared between WKY and SHR. The magnitude of contraction and MLC_{20} phosphorylation were higher in SHR than WKY. MLC_{20}

phosphorylation level was measured with three different methods; urea-glycerol minigel electrophoresis with total MLC_{20} antibody, SDS-PAGE with specific antibodies for mono- (Ser-19) and diphosphorylated (Ser-19/Thr-18) MLC_{20}[40,41] and immunofluorescence staining with confocal microscope. In three different methods, the same result was obtained. It suggests that the increase of the calyculin A-induced contraction in SHR is concerned with elevation of mono- and diphosphorylated MLC_{20}.

It is well known that agonist-induced contraction and myogenic tone are higher in SHR than WKY, which is mainly caused by MLC_{20} phosphorylation at Ser-19[39,42]. This is considered due to the increased MLCK activity and increased Ca^{2+} sensitization. However, in the pathological condition such as vasospasm, not only monophosphorylation of MLC_{20} but also diphosphorylation of MLC_{20} was significantly increased[34-36]. Essential hypertension which is the same status of SHR is known to be responsible for many cardiovascular diseases[43]. Among many causes to develop these diseases, increased total peripheral resistance is one of the key hemodynamic abnormalities[43,44]. Increased total peripheral resistance is regarded to be associated with the increase of ATPase activity in myosin. Diphosphorylated MLC_{20} can increase myosin ATPase activity three times more than monophosphorylated MLC_{20}[23,31,32]. Thus, even small amount of diphosphorylated MLC_{20} may be able to play an important role in increased total peripheral resistance and hypertension. As mentioned above, in vasospasm models, increased diphosphorylated MLC_{20} is clearly shown which is followed by narrowing of blood vessels[34-36]. Therefore, calyculin A-induced contraction and MLC_{20} phosphorylation may be associated with hyper-contractility in SHR.

Since Ca^{2+}-independent and microcystin-LR-induced contraction and MLC_{20} phosphorylation was first demonstrated by Kureishi et al[45], a number of Ca^{2+}-independent kinases have been subsequently shown to phosphorylate MLC_{20} in vitro. ROCK[46], PKC[6], MAPK-activated protein kinase-1b (RSK-2)[47], mitogen-activated-protein kinase (MAPK)-activated protein kinase-2 (MAPKAPK2)[48], ILK[27] and ZIPK[9,49] were suggested to be able to phosphorylate MLC_{20} both at Ser-19 and Thr-18 in the absence of Ca^{2+}. However, it was revealed that ROCK, RSK-2 and MAPKAPK2 phosphorylation of MLC_{20} is restricted to Ser-19 and PKC can

phosphorylate neither Ser-19 nor Thr-18[25]. ZIPK and ILK can phosphorylate MLC_{20} without Ca^{2+} in smooth muscle and they have no preference between Ser-19 and Thr-18 of MLC_{20}[50]. Therefore, ZIPK and ILK have emerged as the most likely candidates for the Ca^{2+}-independent MLCK in smooth muscle[9,28]. To figure out its nature and the exact role as Ca^{2+}-independent MLCK, inhibitors for ZIPK and ILK are necessary. However, only ZIPK inhibiting peptides, SM-1 and AV-25 were developed until now. Both are made from the auto-inhibitory domain of MLCK which shares similar sequences with ZIPK. SM-1 was confirmed as a useful material to distinguish ZIPK from ILK because it was proved no inhibitory effect to ILK[51]. In this experiment, SM-1 inhibited calyculin A-induced contraction almost totally in β-escin permeabilized rat mesenteric artery. It was confirmed with scrambled peptide of SM-1, which showed no effect on calyculin A-induced contraction. Furthermore, unlike ILK expression which was similar between WKY and SHR, ZIPK (54 and 32kDa) expression was greater in SHR than WKY. It has been shown that a 32kDa form of ZIPK expressed in smooth muscle co-localizes with actin filaments and plays a role in the regulation of smooth muscle contraction[51]. A 54kDa form of ZIPK is also expressed in smooth muscle and activated by prostaglandin $F_2\alpha$ stimulation[26]. Taken together, it suggests that ZIPK may play an important role in SHR as Ca^{2+}-independent MLCK.

ZIPK regulates smooth muscle contraction via three different pathways. It can phosphorylate MYPT1 Thr-696[8,52] and Thr-850[53], CPI-17 Thr-38[9,13,52] and MLC_{20} Ser-19/Thr-18 directly[9,52]. To determine how ZIPK induces augmented MLC_{20} phosphorylation by calyculin A in SHR, calyculin A-induced phosphorylation of MYPT1 Thr-697, Thr-855 and CPI-17 Thr-38 were compared between WKY and SHR. Phosphorylation of MYPT1 and CPI-17 was increased in both WKY and SHR. However, only MYPT1 Thr-697 phosphorylation was significantly more increased in SHR than WKY.

MYPT1 Thr-696 and Thr-850 and CPI-17 Thr-38 can be phosphorylated by several kinases, such as ROCK[54,55], ILK[10,56] and ZIPK[9,16]. In hypertension, ROCK contributes to increase contractility[57] by the phosphorylation of MYPT1 at Thr-696 and Thr-850 and it has a preference for MYPT Thr-850 compared to Thr-696[53]. On the contrary to ROCK, ZIPK has a strong preference for MYPT1 Thr-696 relative to Thr-850[53]. This supports the results that calyculin A-induced phosphorylation of

MYPT1 at Thr-697 is greater in SHR. Therefore, ZIPK may be involved in hyper-contractility of SHR via phosphorylation of MYPT1 at Thr-697. Additionally, ZIPK can phosphorylate MLC_{20} in vitro directly[9,24]. Thus, ZIPK-induced direct MLC_{20} phosphorylation may take a part in calyculin A-induced contraction in SHR mesenteric artery. Whether ZIPK phosphorylates MLC_{20} directly and contributes to hypertension, it remains to be determined.

In summary, these results strongly suggest that Ca^{2+}-independent and calyculin A-induced contraction and MLC_{20} phosphorylation are enhanced in SHR compared to WKY. ZIPK may be the key candidate for Ca^{2+}-independent MLCK in rat mesenteric artery. Furthermore, the increase of MYPT1 Thr-697 phosphorylation elicited by highly expressed ZIPK in SHR plays an important role in the enhanced calyculin A-induced contraction and MLC_{20} phosphorylation in SHR.

Ⅴ. *Conclusion*

To determine the role of Ca^{2+}-independent MLCK in hypertension, the contractile force, phosphorylation of MLC_{20} and phosphorylation of MYPT1 and CPI-17 evoked by calyculin A in β-escin permeabilized mesenteric artery at pCa9.0 were compared between WKY and SHR. Calyculin A-induced contraction was significantly greater in SHR than WKY and the augmentation of contraction in SHR was concerned with the increase in mono- and diphosphorylation of MLC_{20}. SM-1, ZIPK inhibiting peptide suppressed calyculin A-induced contraction. ZIPK expressed abundantly in rat mesenteric artery and its expression level was higher in SHR than WKY. Phosphorylation of MYPT1 at Thr-697, Thr-855 and CPI-17 at Thr-38 was increased by calyculin A in both WKY and SHR. However, calyculin A-induced MYPT1 phosphorylation at Thr-697, not Thr-855, was greater in SHR compared to WKY. CPI-17 phosphorylation at Thr-38 was similar in both WKY and SHR. These results suggest that Ca^{2+}-independent and calyculin A-induced contraction is enhanced in β-escin permeabilized mesenteric artery in SHR, which is correlated with increased mono- and diphosphorylation of MLC_{20}. It is further elucidated that ZIPK may play a role as the Ca^{2+}-independent MLCK in rat mesenteric artery. Increased phosphorylation of MYPT1 at Thr-697 may be involved in enhanced calyculin A-induced contraction and MLC_{20} phosphorylation in SHR.

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Abstract (in Korean)

본태성 고혈압 쥐에서 Ca^{2+} 비의존성 MLCK에 의한 myosin 인산화와 수축의 증가

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조 영 은

평활근의 수축은 20kDa myosin light chain(MLC_{20})의 인산화 정도에 따라 결정된다. 세포 내 Ca^{2+}에 의해 myosin light chain kinase(MLCK)이 활성화 되거나 Ca^{2+}과는 관계 없이 조절되는 myosin light chain phosphatase(MLCP)가 억제되었을 때 MLC_{20}는 인산화되어 수축을 일으킨다. 최근 이 두 가지 조절 기전 이외에, Ca^{2+}에 관계없이 MLC_{20}을 인산화시키는 MLCK도 존재함이 밝혀졌다. 이는 평활근 조직의 막투과도를 증가시켜 세포 내외의 Ca^{2+}을 제거한 후, 단백질 탈인산화 효소 억제제인 microcystin-LR을 처리하였을 때 수축이 일어나며, 이것이 비특이적 kinase 억제제인 staurosporine에 의해서 억제되는 실험결과를 통해 확인되었다. 이러한 Ca^{2+} 비의존성 MLCK에 의한 수축은 MLC_{20}의 Ser-19의 인산화뿐 아니라 Ser-19/Thr-18의 인산화를 동반한다. MLC_{20}의 Ser-19/Thr-18 인산화는 혈관 평활근의 과수축과 관련되어 있음이 혈관 경련(vasospasm) 등의 혈관 질환 모델에서 밝혀져 있다. 본태성 고혈압 또한 혈관의 과수축이 일어나는 대표적 혈관 질환이지만, MLC_{20}의 Ser-19/Thr-18 인산화가 이에 관여하는지에 대해서는 아직 알려져 있지 않다.

그러므로 본 실험에서는 본태성 고혈압에서 Ca^{2+} 비의존성 MLCK의 역할을 규명하고자, 본태성 고혈압 쥐(SHR)와 정상 혈압 쥐(WKY)에서 Ca^{2+} 비의존성 MLCK에 의한 수축, MLC_{20}의 인산화, 그리고 여러 신호조절 물질들의 변화를 비교하였다.

쥐의 장간막 동맥을 β-escin으로 막투과도를 증가시켜 세포 내외의 Ca^{2+}을 제거한 후, 1μM calyculin A(1,2A형 탈인산화 억제제)를 처리하였다. 점진적인 수축이 일어나 20~30분 후 안정화 되었으며, 비특이적 kinase 억제제인 staurosporine에 의해서만 억제되었다. 이 수축은 WKY보다 SHR에서 더 크게 나타났으며, 이는 SHR에서 증가된 MLC_{20}의 인산화(Ser-19과 Ser-19/Thr-18의 인산화)와 관련됨을 알 수 있었다. Ca^{2+} 비의존성 MLCK의 후보물질 중 하나인 ZIPK의 억제 펩타이드, SM-1은 calyculin A에 의한 수축을 유의하게 억제하였고, 실제로 ZIPK는 SHR의 장간막 동맥에서 더 많이 발현되어 있음이 확인되었다. Calyculin A에 의한 수축에서는 MYPT1 Thr-697, Thr-855과 CPI-17의 Thr-38의 인산화가 관찰 되었는데, 이 중 MYPT1 Thr-697의 인산화만이 SHR에서 유의하게 증가하였다.

이상의 결과들을 통해, 막투과도를 증가시킨 본태성 고혈압 쥐의 장간막 동맥에서 Ca^{2+} 비의존성 MLCK에 의한 수축이 더 크게 나타나며, 이는 MLC_{20} Ser-19과 Ser-19/Thr-18의 인산화 증가로 인해 일어나는 것을 알 수 있었다. 쥐의 장간막 동맥에서 작용하는 Ca^{2+} 비의존성 MLCK는 ZIPK인 것으로 생각되며, ZIPK는 MYPT1의 Thr-697의 인산화를 통해 본태성 고혈압 쥐에서 증가된 calyculin A에 의한 수축과 MLC_{20} 인산화를 일으킬 것으로 예상된다.

핵심되는 말 : Ca^{2+} 비의존성 MLCK, ZIPK, 고혈압, MLC_{20} Ser-19/Thr-18 인산화