Cardiovascular Research (2008) **80**, 346–353
doi:10.1093/cvr/cvn211

Enhanced pulsatile pressure accelerates vascular smooth muscle migration: implications for atherogenesis of hypertension

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Received 12 January 2008; revised 2 July 2008; accepted 28 July 2008; online publish-ahead-of-print 7 August 2008

Time for primary review: 21 days

KEYWORDS
Pulsatile pressure;
Vascular smooth muscle cell;
Migration;
Calcium

Aims Clinical studies have suggested that pulsatile pressure is an independent risk factor for atherosclerosis. However, it is unknown whether enhanced pulsatile pressure *per se* directly accelerates vascular smooth muscle cell (VSMC) migration, an important process of atherosclerosis.
Methods and results Using our original Pressure-loading system with a Boyden chamber, we examined the direct effects of variable pressures and pulse rates on migration of rat aortic VSMCs *in vitro*. High pulse pressure (180/90 mmHg, pulsatile vs. 180 mmHg, static), high mean pressure (180/90 vs. 90/0 mmHg, with the same pulse pressure), wide pulse pressure (190/110 vs. 170/130 mmHg, with the same mean pressure), and high pulse rate (120 vs. 40 per min) significantly accelerated the VSMC migration (1.35, 2.38, 1.38 and 1.27-fold, respectively). The increase in intracellular calcium levels measured by fura-2/AM fluorescence was proportional to the magnitude of pressure loaded. The pressure-promoted VSMC migration was significantly inhibited by a phospholipase-C inhibitor U-73122 or a calmodulin inhibitor W-7. Inositol 1,4,5-trisphosphate receptor blockers 2-aminoethoxydiphenyl borate or xestospongin-C significantly inhibited the VSMC migration, whereas a ryanodine receptor blocker ryanodine had no effects. Furthermore, a calcium channel blocker (CCB), azelnidipine, and an angiotensin type-1 receptor blocker, olmesartan, also significantly inhibited the VSMC migration.
Conclusion These results provide direct evidence for the pro-atherogenic effects of enhanced pulsatile pressure and also suggest that the anti-atherogenic actions of CCBs and angiotensin type-1 receptor blockers are mediated in part by their direct inhibitory effects on VSMC migration in addition to their anti-hypertensive effects.

1. Introduction

Hypertension is one of the most important risk factors for the initiation and development of atherosclerosis.[1] Several clinical trials have shown that among the several haemodynamic factors, pulsatile pressure is an independent risk factor for cardiovascular diseases.[2–4] Pulsatile pressure is a pressure with pulsation that has various factors such as mean pressure, pulse pressure (the gap between the highest and the lowest values of the pulsatile pressure), and pulse rate. Indeed, the Framingham Heart Study has demonstrated that hypertension-related morbidity and mortality corresponded best with pulse pressure.[5] The major determinant of pressure-induced remodelling *in vivo* may be a magnitude and/or a mode of pulsatile pressure rather than a simple elevation of mean arterial pressure.[6,7] However, the detailed mechanisms for the atherogenic effects of pulsatile pressure remain to be examined.[5,6]

Proliferation and migration of vascular smooth muscle cells (VSMCs) are critical processes for the development of atherosclerosis. These responses of VSMCs are regulated by several factors, among which mechanical stress may play a major role.[8] Indeed, it was previously demonstrated that increased static pressure promotes VSMC proliferation.[9] We also have recently demonstrated that enhanced static pressure accelerates VSMC migration.[10] However, it remains to be elucidated whether enhanced pulsatile pressure *per se* accelerates VSMC migration.

There are several signal transduction pathways involved in cell migration, including small G-proteins, Rho-kinase, actin-binding proteins, and myosin II motors, where several humoral factors accelerate cell migration by modulating intracellular calcium level.[11,12] Thus, the effects of

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pulsatile pressure on cell migration are likely to be mediated by intracellular calcium levels.

In the present study, we thus directly addressed these important issues, using our original *in vitro* system with a Boyden chamber system that enables us to examine the effects of variable types and magnitudes of pulsatile pressure on VSMC migration.

2. Methods

The present study conforms with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the ethical committee on basic experiments of Tohoku University Graduate School of Medicine.

2.1 Isolation and culture of vascular smooth muscle cells

A primary culture of VSMCs was performed as previously described.[10,13] Thoracic and abdominal aortas were excised from Wistar rats weighing 200 g under pentobarbital anaesthesia (50 mg/kg IP). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C, and VSMCs of fifth to 10th passage were used. The cells were identified as VSMCs by immunocytochemistry with a monoclonal anti-VSMC α-actin antibody.[14]

2.2 Pressure-loading apparatus

To selectively examine the effects of loading pressure on VSMC migration *in vitro*, we developed an original pressure-loading apparatus equipped with an intra-aortic balloon pump (IABP) and a Boyden chamber (*Figure 1*). Briefly, a custom-designed glass bottle was used as a pressure-loading apparatus, in which the membrane of a Boyden chamber was filled with VSMCs and a sufficient amount of saline was added at the bottom for humidification. The glass bottle was then packed with a silicon plug that was connected to an IABP (System 90T, Datascope, Tokyo, Japan) with a manometer. Variable pulsatile pressures were produced by the insufflation of air from IABP in this closed circuit. As a control, the same type of closed circuit with the same amount of air was prepared. The loading pressure was monitored by a connected manometer, and was displayed on the IABP panel. Variable types and magnitudes of pulsatile pressure were created by the IABP machine. The duration of inflation and deflation to the balloon was equivalent. The frequency of insufflation was 60 per min unless specified.

To estimate cell damage and/or cell proliferation by mechanical pressure, VSMCs were incubated under pulsatile pressure on fibronectin-coated dishes for 6 h. After staining with trypan blue, the number of viable cells was counted. After loading the pulsatile pressure, the number of VSMCs was not significantly changed, confirming the viability of those cells. The pore size of Boyden chambers was not significantly changed at 6 h after loading pressure.

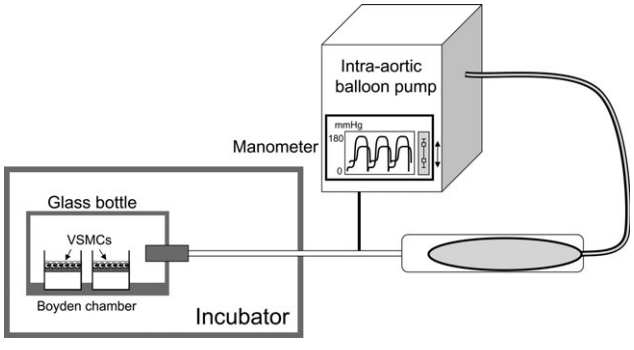

Figure 1 Schema of the original apparatus with a Boyden chamber. This apparatus enables us to generate variable types and magnitudes of pulsatile pressure that are loaded on vascular smooth muscle cells.

2.3 Boyden chamber assay

Migration assay across the membrane was performed with modified Boyden chambers as previously described.[10,15] Briefly, the membrane of the upper chamber with pores of 8 μm in diameter (BD Falcon, San Jose, CA, USA) was pre-coated with fibronectin (1 $\mu g/cm^2$). The upper chamber containing VSMCs (160 000 cells/cm^2) in Hanks medium 199 (M199) with 5% FBS was set on the lower chamber that was also filled with M199 containing 5% FBS. The VSMCs were pre-incubated without adding pressure for 1 h to adhere on chamber membrane. The chamber was then exposed to variable types of pressure by the above IABP system for 6 h at 37°C, because the effect on cell migration was most evident at 6 h among several time points in the preliminary experiment. After the cells on the upper surface of the membrane were removed with a cotton swab, the cells on the underside of the membrane were stained with Giemsa and counted by light microscopy at a magnification of $\times$40. VSMC migration activity was calculated as the mean number of migrated cells observed in four high-power fields ($\times$100) and was obtained as a mean value of the four measurements.

An inhibitor or vehicle was administered to the medium 1 h after inserting the cells into the Boyden chamber, and the chamber was exposed to the pressure 1 h later.

2.4 Measurement of intracellular calcium levels

To analyse the detailed mechanism of pressure-promoted cell migration, intracellular calcium levels were measured by the fura-2/AM method,[16] using the optical systems for fluorescence signal detection.[17,18] VSMCs, cultured semi-confluently on a flask, were loaded with 2 μmol/L fura-2/AM. After the fura-2 was loaded, the cells were rinsed off and flasks were directly connected with pressure-loading circuit and background autofluorescence was measured. During loading pressure, the cells were excited on the stage of an inverted microscope (Nikon, Tokyo, Japan) at wavelengths of 340 and 380 nm with a Xenon lamp, and emission was recorded at 505 nm. After background autofluorescence from unloaded VSMCs was subtracted at the end of each experiment, the ratio of emitted fluorescence (F340/F380) was calculated. The fluorescence intensity was measured at a rate of 20 points/s. Inhibitors were pre-incubated for 1 h before pressure loading and measurements.

2.5 Materials

DMEM, M199, 2-aminoethyl diphenylborinate (2-APB), ryanodine from *Ryania speciosa*, U-73122, and monoclonal anti-VSMC α-actin antibody were obtained from Sigma Chemical Co. (St Louis, MO, USA). FBS was obtained from Gibco Laboratories (Grand Island, NY, USA). *O,O′*-bis (2-aminophenyl) ethyleneglycol- *N,N,N′,N′*- tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) and fura-2/AM were obtained from Dojindo Laboratories (Kumamoto, Japan). Xestospongin-C and Giemsa were obtained from Merck (San Diego, CA, USA). *N*-(6-aminohexyl)- 5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) and *N*-(6-aminohexyl)-1- naphthalenesulphonamide hydrochloride (W-5) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Fibronectin was obtained from Cosmo Bio Co. (Tokyo, Japan). Azelnidipine and olmesartan (RNH-6270) were kindly provided by Sankyo Pharmaceutical Co. (Tokyo, Japan).

2.6 Statistical analysis

The experiments of migration were performed in triplicate. The results were expressed as means $\pm$ SEM, and were analysed by

unpaired *t*-test with SPSS 11.0J (Chicago, IL, USA). Intracellular calcium levels were analysed by paired *t*-test with the same software. Differences between more than three groups were analysed by Analysis of variance. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Effects of pulsatile pressure on vascular smooth muscle cell migration

To examine the effects of pulsatile pressure (mean value, pulse width, and frequency), variable types and magnitudes of pressure were created by the IABP machine. As compared with static pressure (180 mmHg, maintained), pulsatile pressure (180/90 mmHg) significantly increased cell migration (*Figure 2A*). Under the same magnitude of pulse pressure, the VSMC migration was significantly enhanced at higher pressure (180/90 mmHg) than at lower pressure (90/0 mmHg) (*Figure 2B*). Furthermore, even with the same mean pressure, the migration was significantly enhanced more at a greater pulse pressure (80 at 190/110 mmHg) than at a smaller pulse pressure (40 at 170/130 mmHg) (*Figure 2C*). Finally, with the same mean and pulse pressure, the VSMC migration was enhanced more at a higher frequency (120 per min) than at a lower frequency (40 per min) (*Figure 2D*).

3.2 Involvement of intracellular calcium-related pathway in the pressure-promoted vascular smooth muscle cell migration

For detailed mechanism of pressure-promoted cell migration, we focused on the intracellular calcium signalling. The VSMC migration was completely inhibited by U-73122 (10 μmol/L), the blocker of phospholipase-C (PLC), under both pressure-free and pressure-loaded (180/90 mmHg, 60 per min) conditions (*Figure 3A*). W-7 (5 μmol/L), a calmodulin blocker, also inhibited the migration under pressure-loaded condition (180/90 mmHg), but not under pressure-free condition, whereas its negative control, W-5 (5 μmol/L), was without effects (*Figure 3B*).

3.3 Effects of inhibitors of inositol 1,4,5-trisphosphate receptor and ryanodine receptor

Then intracellular calcium levels were measured by the fura-2/AM method. Intracellular calcium level, evaluated by F340/380 ratio, was significantly increased in response to both static and pulsatile pressure (*Figure 4*) and correlated with the increase in cell migration (*Figure 2*). Both the inositol 1,4,5-trisphosphate (IP3) receptor and ryanodine receptor mediate calcium release from intracellular store sites.[19] IP3 receptor blockers, 2-APB (100 μmol/L) and xestospongin-C (10 μmol/L), did not affect intracellular calcium level under pressure-free condition, but abolished

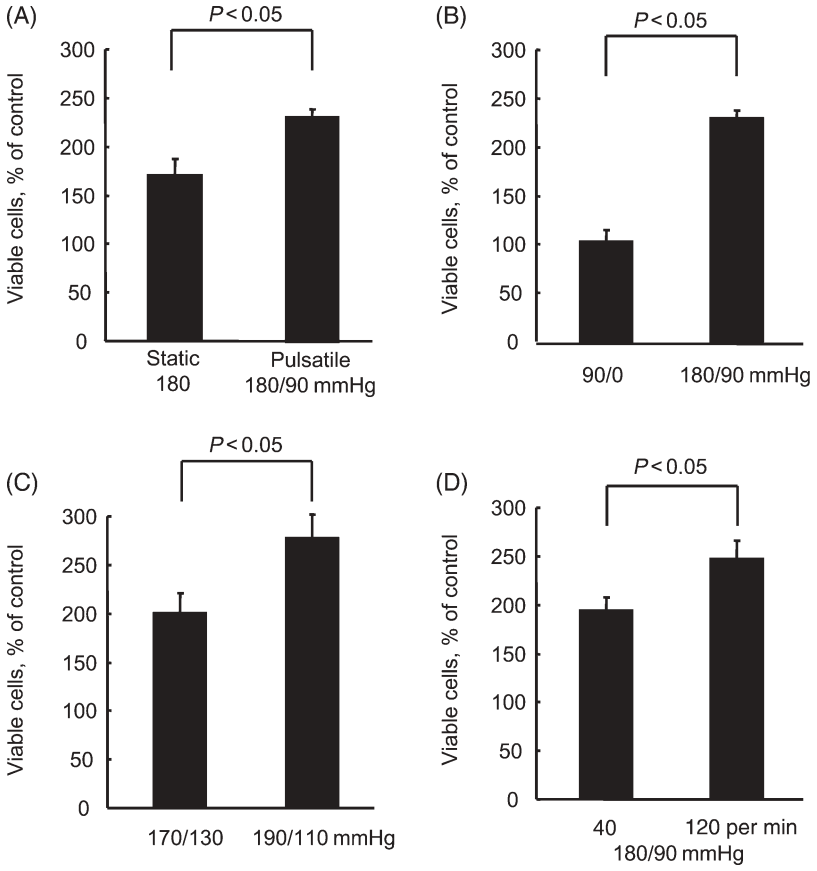

Figure 2 Pulsatile pressure enhances vascular smooth muscle cell (VSMC) migration. Pulsatile pressure (*A*), high mean pressure (*B*), wide pulse pressure (*C*), and high frequency (*D*) accelerated VSMC migration of rat aortic VSMCs. Data from cells under no pressure serve as control. Each graph shows relative increase in the number of migrated cells under various pulsatile pressures as compared with no pressure. Results are expressed as means ± SEM ($n = 7$ each).

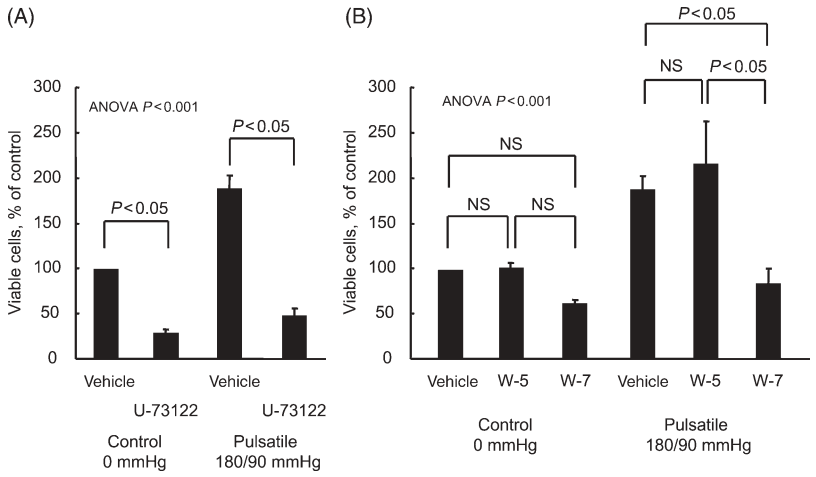

Figure 3 Effect of inhibitors of intracellular calcium levels on vascular smooth muscle cell (VSMC) migration. VSMC were pre-incubated with 10 μmol/L U-73122 (a phospholipase-C inhibitor), 10 μmol/L W-7 (a selective calmodulin inhibitor) or W-5 (its negative control) for 1 h. U-73122 (*A*) significantly inhibited the VSMC migration under both no-pressure and pulsatile pressure condition (180/90 mmHg, 60 per min). W-7 (*B*) also significantly inhibited the VSMC migration under pulsatile pressure, but not under no-pressure condition. Each graph shows relative increase in the number of migrated cells under various pulsatile pressures as compared with no pressure. Results are expressed as means ± SEM ($n = 7$ each).

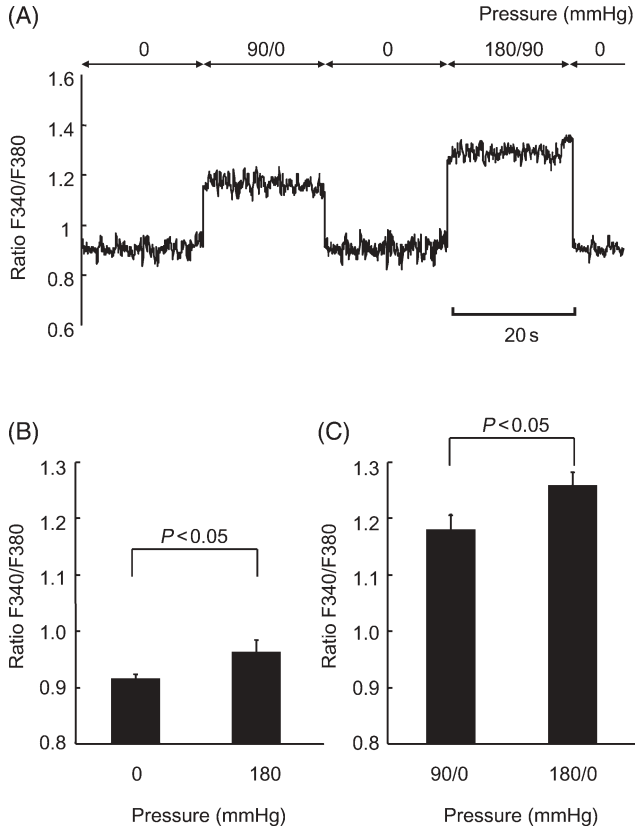

Figure 4 Measurement of intracellular calcium level. Representative tracing of calcium fluorescence in response to pulsatile pressure (*A*) showing that F340/F380 ratio was increased in a stepwise manner in response to the pulsatile pressure. Quantitative analysis of intracellular calcium in response to pressure shows that static pressure (*B*) and pulsatile pressure (*C*) significantly increased intracellular calcium levels as evaluated by the ratio of F340/F380. Results are expressed as means ± SEM ($n = 7$ each).

the increase in intracellular calcium level at 180/90 mmHg (*Figure 5A*). In contrast, ryanodine (10 μmol/L), a ryanodine receptor blocker, had no effects under both pressure-free and pressure-loaded conditions. The inhibitory effects of 2-APB (100 μmol/L) and xestospongin-C (10 μmol/L) on the VSMC migration were consistent with their inhibitory effects on intracellular calcium levels (*Figure 5B*). Ryanodine (10 μmol/L) also had no effects on VSMC migration as was the case with intracellular calcium levels (*Figure 5C*).

3.4 Effects of a calcium channel blocker and an angiotensin type-1 receptor blocker

Finally, the inhibitory effects of clinical concentrations of calcium channel blocker (CCB) and angiotensin receptor blocker (ARB) on pressure-promoted cell migration were examined. Azelnidipine (1 μmol/L), an L-type CCB, did not affect VSMC migration or intracellular calcium levels under pressure-free condition, but significantly inhibited pressure-promoted VSMC migration and the increase in intracellular calcium level at 180/90 mmHg (*Figure 6A*). Olmesartan (10 nmol/L), an angiotensin II type receptor blocker, did not affect VSMC migration or intracellular calcium levels under pressure-free condition, but significantly inhibited pressure-promoted VSMC migration without altering intracellular calcium levels at 180/90 mmHg (*Figure 6B*).

4. Discussion

The major finding of this study is that pulsatile pressure (mean pressure, pulse pressure, and pulse rate) directly promotes migration of rat aortic VSMCs *in vitro*, for which intracellular calcium release via IP3 receptors may be involved. Both azelnidipine and olmesartan abolished pressure-promoted VSMC migration, indicating that the anti-atherogenic action by these drugs may be mediated by inhibition of pressure-promoted VSMC migration, in addition to their anti-hypertensive effect.

4.1 Role of vascular smooth muscle cell migration in the pathogenesis of atherosclerosis

VSMC migration is regulated by several cytokines, such as platelet-derived growth factor (PDGF) and angiotensin II.[8] However, it remains to be examined whether mechanical stresses affect VSMC migration. The present study provides

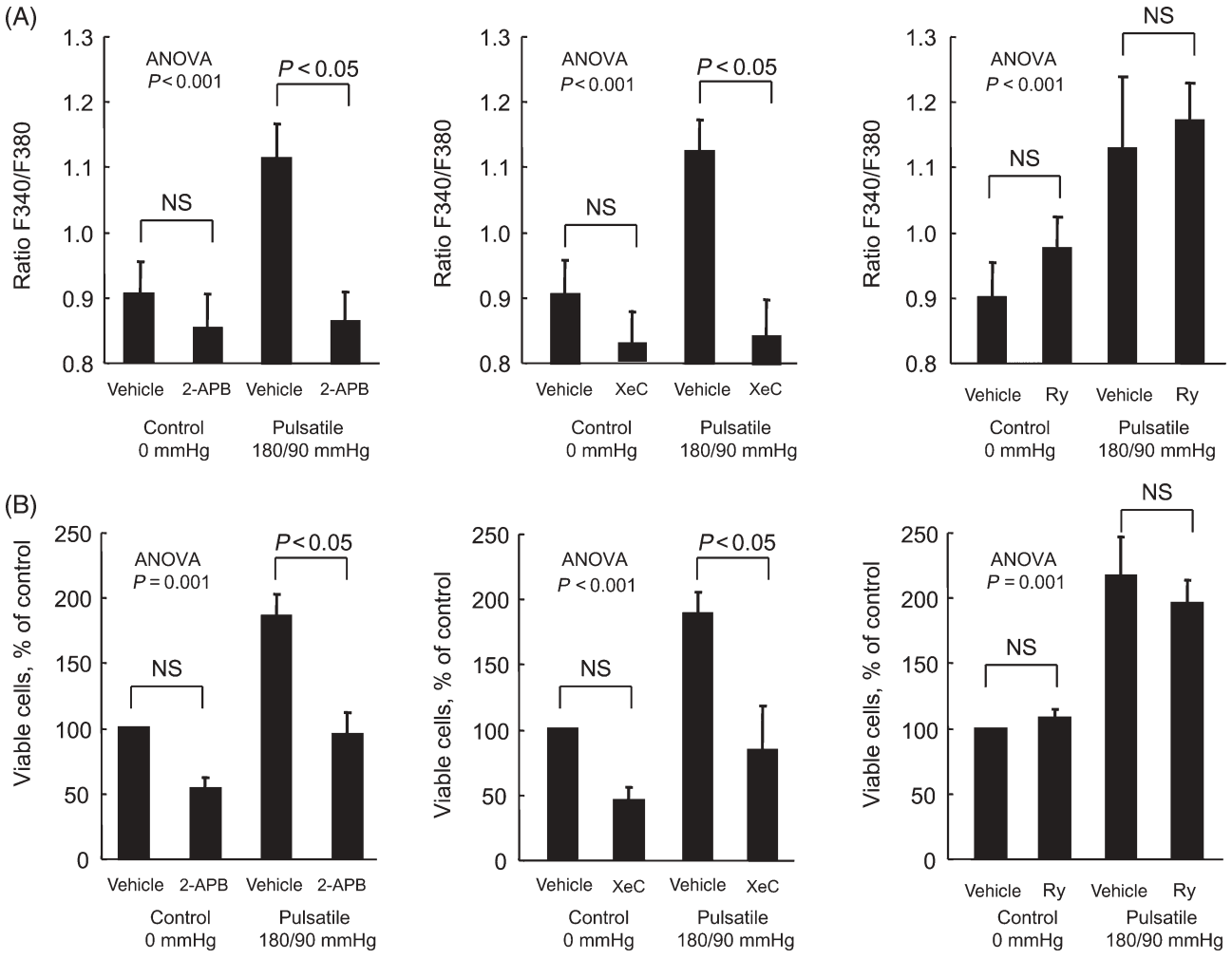

Figure 5 Effects of inositol 1,4,5-trisphosphate (IP3) blockers and ryanodine blocker on vascular smooth muscle cell (VSMC) migration. VSMC were pre-incubated with 100 μmol/L 2-aminoethyl diphenylborinate (2-APB), 10 μmol/L xestospongin-C (a IP_3 receptor blocker), 10 μmol/L ryanodine (ryanodine channel blocker) for 1 h. 2-APB and xestospongin-C, significantly inhibited the pressure-promoted increase in intracellular calcium levels (*A*) and VSMC migration (*B*), whereas ryanodine was without effect. Data from cells under no pressure serve as control in (*B*). Results are expressed as means ± SEM ($n = 7$ each). XeC, xestospongin-C; Ry, ryanodine.

the first evidence for the direct enhancing effects of pulsatile pressure on VSMC migration.

In the present study, VSMC migration was enhanced by the increase in each component of pulsatile pressure (static pressure, mean pressure, pulse pressure, and frequency). This is consistent with the clinical observations that not only high mean pressure but also high pulse pressure and heart rate are associated with high mortality and morbidity in patients with hypertension.[2–7] We also showed that high pulse rate accelerates VSMC migration, suggesting that heart rate also influences the development of atherosclerosis, a consistent finding with the previous clinical studies.[20] The present results also indicate that VSMCs are able to sense not only the magnitude but also the different types of pressure. Taken together, these results suggest that pulsatile pressure-related mechanical stresses directly affect VSMC migration.

4.2 Intracellular mechanisms of pressure-promoted vascular smooth muscle cell migration

In the present study, both static and pulsatile pressure increased intracellular calcium level, correlate with the increase in cell migration, and the pressure-promoted migration was inhibited by a PLC inhibitor (U-73122) and a calmodulin blocker (W-7). PLC, which is activated by several receptors, then up-regulates IP3, which binds to IP3 receptor of sarcoplasmic reticulum to promote the influx of calcium from the intracellular calcium store sites.[21,22] Increased intracellular calcium then binds to calmodulin that plays an important role in cell migration.[23,24] These results indicate that intracellular calcium signalling is one of the critical pathways for pressure-promoted VSMC migration.

Intracellular calcium release from sarcoplasmic reticulum is primarily mediated by IP3 and ryanodine receptors.[19,22] It was reported that vortex-mediated mechanical stress was modulated by IP3-mediated calcium release.[25] The present study demonstrates that the pressure-promoted increase in intracellular calcium level is achieved through IP3-mediated calcium release from the intracellular calcium store and plays an important role in the pathogenesis of atherosclerosis in hypertension.

It is widely known that calcium is a critical cationic second messenger for many cellular processes including VSMC migration.[26] Several humoral factors, such as PDGF and angiotensin II, regulate cell migration through intracellular calcium signalling pathways.[27–30] PDGF accelerates cell

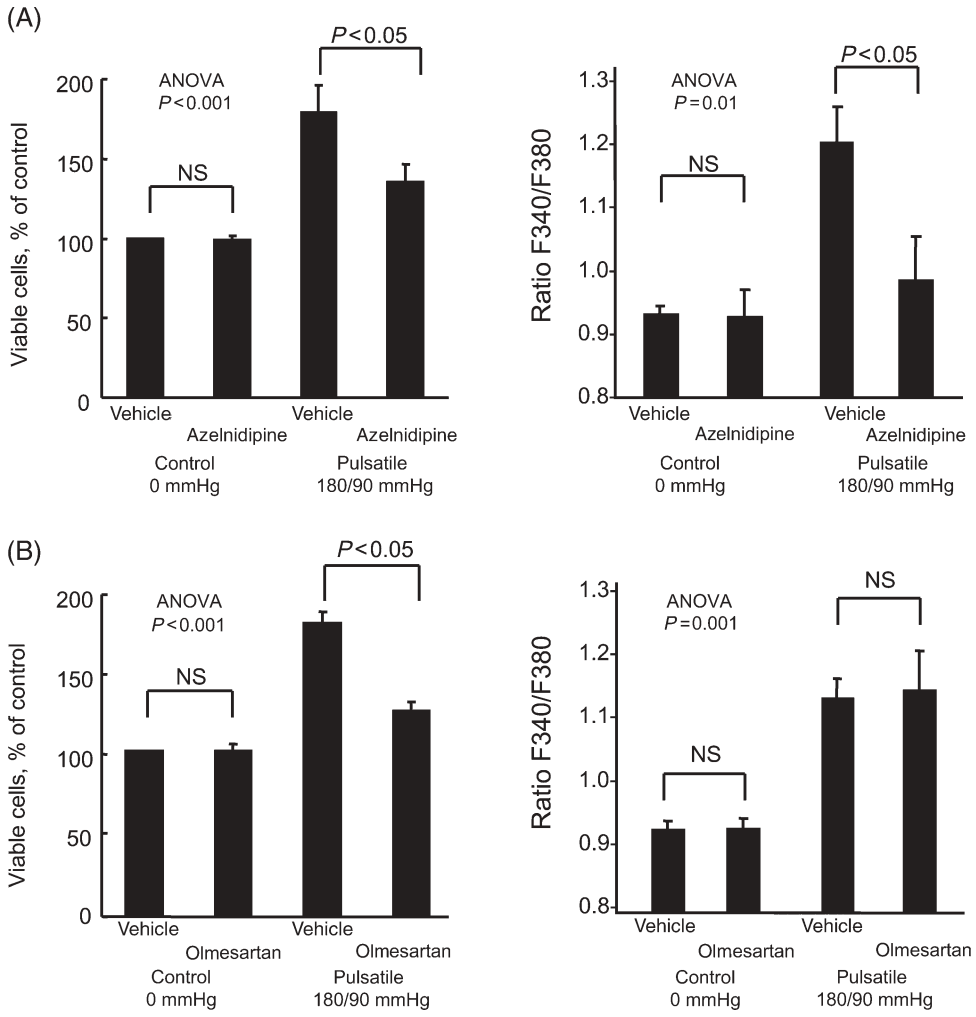

Figure 6 Effect of a calcium channel blocker (CCB) and an angiotensin type-1 receptor blocker on pressure-promoted vascular smooth muscle cell (VSMC) migration and calcium increase. Azelnidipine (1 μmol/L), a CCB, suppressed both VSMC migration and increase in intracellular calcium levels in response to pulsatile pressure (*A*). Olmesartan, angiotensin type-1 receptor blocker (10 nmol/L), suppressed the pressure-promoted VSMC migration, but did not affect intracellular calcium levels (*B*). Data from cells under no pressure serve as control. Results are expressed as means ± SEM ($n = 7$ each).

migration through activation of PLC, the increase in intracellular calcium release from sarcoplasmic reticulum, and calcium–calmodulin pathway.[31] The present results suggest that pressure-promoted signal transductions for VSMC migration resemble those induced by angiotensin II and PDGF. One possibility is that pressure might induce the formation of angiotensin II and/or its release from cells. Another report revealed that mechanical stress activates angiotensin II type-1 receptor (AT1R) without the involvement of angiotensin II.[32] This point remains to be examined in future studies.

Although we have recently reported that static pressure enhances cell migration, for which Rho-kinase may be involved,[10] the detailed mechanism remains to be elucidated. Stretch modulation of VSMC signalling pathway has been recognized,[33] however, little is known about the mechanisms for cell signalling induced by pressure itself. In our pressure-loading apparatus, the loading pressure would not induce stretch stimulation on VSMC. It is thus conceivable that an unidentified mechanosensor(s) senses not only loading pressure but also relaxation after loading pressure. As compared with static pressure or high mean pressure, pulsatile pressure would more strongly activate a mechanosensor(s) through pressure loading and relaxation, where wide pulse pressure and higher frequency would further accelerate VSMC migration.

4.3 Effects of calcium channel blockers and angiotensin receptor blockers

In the present study, both azelnidipine and olmesartan effectively inhibited the pressure-promoted VSMC migration. However, the mechanisms for their inhibitory effects may be different because azelnidipine suppressed both pressure-promoted VSMC migration and increase in intracellular calcium levels, whereas olmesartan only inhibited the former response alone. L-type calcium channels mediate calcium influx from extracellular space, when triggered by the calcium release from sarcoplasmic reticulum.[34] The inhibition of pressure-promoted VSMC migration by azelnidipine may thus result from the suppression of calcium influx at the level of L-type calcium channels.

On the other hand, the effects of ARB on the cell migration may be complex. Although it was reported that AT1R could be one of the mechanosensers,[32] the present study demonstrates that olmesartan suppresses the pressure-promoted VSMC migration without altering intracellular calcium levels. AT1R is known to be coupled to

various downstream signalling pathways in addition to calcium signalling.[35] For example, Rho-kinase, which is also activated by AT1R, is substantially involved in cell migration[36] and we have recently demonstrated that static pressure enhances VSMC migration partly through Rho-kinase activation.[10] Since the blockade of AT1R suppresses all the downstream signalling pathways, the inhibitory effect of olmesartan on VSMC migration is likely mediated by calcium-independent mechanisms(s).

4.4 Clinical implications

The present study demonstrates that enhanced pulsatile pressures promote VSMC migration through intracellular calcium handling processes and suggests that the anti-atherogenic actions of CCBs and ARBs are mediated, at least in part, by their inhibitory effects on VSMC migration in addition to their anti-hypertensive effects. Thus, pulsatile pressure is an important therapeutic target in the treatment of hypertensive cardiovascular diseases.

Funding

This work was supported in part by the grants-in-aid (Nos. 16209027, 16659192) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

Acknowledgements

We are grateful to Fumie Hase for excellent technical assistance for this study.

Conflict of interest: none declared.

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