

Akt/eNOS and MAPK Signaling Pathways Mediated the Phenotypic Switching of Thoracic Aorta Vascular Smooth Muscle Cells in Aging/Hypertensive Rats

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Summary

Considerable evidence demonstrates that phenotypic switching of vascular smooth muscle cells (VSMCs) is influenced by aging and hypertension. During phenotypic switching, VSMCs undergo a switch to a proliferative and migratory phenotype, with this switch being a common pathology in cardiovascular diseases. The aim of this study was to explore the joint influence of age and hypertension on thoracic aortic smooth muscle phenotypic switching and the balance of Akt and mitogen-activated protein kinase (MAPK) signaling during this switch. Different ages of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) were used to establish hypertension and aging models. The phenotypic state was determined by detecting the marker proteins α -SM-actin, calponin, and osteopontin (OPN) *via* immunohistochemical staining and Western blot. Signaling proteins associated with the Akt and MAPK pathways were detected in rat thoracic aorta using Western blot. Both aging and hypertension caused a decrease in contractile (differentiated) phenotype markers (α -SM-actin and calponin), while the synthetic (proliferative or de-differentiated) phenotype marker was elevated (OPN). When combining hypertension and aging, this effect was enhanced, with Akt signaling decreased, while MAPK signaling was increased. These results suggested that VSMCs phenotype switching is modulated by a balance between Akt and MAPK signaling in the process of aging and hypertension.

Key words

Aging • Hypertension • Vascular smooth muscle cell • Phenotypic switching

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Introduction

Aging and hypertension, which are critical risk factors contributing to cardiovascular diseases, are associated morphological and functional vascular changes characterized by endothelial dysfunction, wall thickening, reduced elasticity, and arterial stiffening. While vascular remodeling is associated with aging, its effects are enhanced by hypertension. Changes in blood pressure are closely related to blood vessel function, with vasomotor regulation mainly impacting the vascular smooth muscle cells (VSMCs). During the cardiac cycle, VSMCs are subjected to significant mechanical strain. While these cells have shown a high degree of plasticity (Bochaton-Piallat and Gabbiani 2005), similarly to myocardial and skeletal muscle cells, the mechanisms behind their phenotypic modulation remains unclear. Encompassed within this plasticity is an ability to alter VSMC differentiation marker profiles, a process termed phenotypic modulation or switching that is often associated with repair or several disease states (Owens *et al.* 2004, Sobue *et al.* 1999). For example, during the early stages of vasculogenesis, VSMCs are highly migratory, undergo very rapid cell proliferation, and undergo phenotypic changes in the vessel wall in response to the degree of mechanical strain, thus enabling

them to adapt to the new conditions.

In VSMCs, phenotypic switching plays a critical role in the pathogenesis of many proliferative cardiovascular diseases, including hypertension, myocardial infarction, and coronary heart disease. During the process of phenotypic switching, protein kinase B (Akt) signaling has been implicated (Goncharova *et al.* 2002, Chol *et al.* 2010, Jeffery and Morrell 2002, Mandegar *et al.* 2004). The Akt pathway plays a critical role in many physiological and pathological processes, to include inflammatory responses, signal transduction, and cell cycle control. The most influential factor responsible for maintaining the differentiated VSMC phenotype is insulin-like growth factor-I (IGF-1), which targets the phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) pathways (Martin *et al.* 2007). A downstream target of Akt is the enzyme endothelial nitric oxide synthase (eNOS), which is responsible for the production of physiological amount of nitric oxide (NO) and serves as a protective agent in a large number of diseases (Li *et al.* 2016, Wu *et al.* 2011). Within the cardiovascular system, endothelial-derived NO plays a pivotal role as a key secondary messenger that regulates physiological and pathological activities (Franceschelli *et al.* 2017). Another important pathway in phenotypic switching is the mitogen-activated protein kinase (MAPK) pathway. MAPK signaling molecules consist of the following protein kinase families: MAPK kinase kinases (MKKKs), MAPK kinases (MKKs), and MAPKs. Of these, extracellular signal-regulated kinase (ERK) and p38 MAPK have been associated with VSMC phenotypic switching, but Jun N-terminal kinase (JNK) has not (Ma and Wells 2014, Xue *et al.* 2016, Harvey *et al.* 2016). Furthermore, Akt, ERK, and p38 MAPK signaling can target platelet-derived growth factor (PDGF), which is known to promote cell proliferation and migration (Hayashi *et al.* 1999). Little is known about whether these signaling alterations interact in the presence of both hypertension and aging, or if they do, if their joint modulation accelerates VSMC phenotypic switching. However, previous findings would suggest that cross-talk does occur between the Akt and MAPK pathways during phenotypic switching.

Accordingly, the primary aims of this study were to investigate if 1) hypertension superimposed with aging promotes VSMC phenotypic switching and 2) whether VSMC phenotypic modulation is associated with a balance between Akt and MAPK signaling pathway. To address these aims, spontaneously hypertensive rats

(SHR) and Wistar-Kyoto rats (WKY) were used. Blood pressure, vascular morphology, contractile proteins, and a synthetic protein were detected in the presence of aging and hypertension, to include examination of the Akt/eNOS, ERK, and p38 MAPK pathways.

Materials and Methods

Animal model and sample preparation

In this study, adult male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) were studied at the ages of 1, 3, 9, and 16 months (Okamoto 1969, Lindsay *et al.* 2016, Li Y. *et al.* 2014). All the rats were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained at a 12:12 h light-dark cycle, with a room temperature of 23-25 °C, humidity level of 40-60 %, and they were provided free access to standard rodent chow and water. Rats were anesthetized with pentobarbitone sodium (50 mg·kg⁻¹, intraperitoneal) and heart weight, body weight, systolic (SBP), diastolic (DBP), and heart rate (HR) were measured in warmed, restrained, conscious rats using the artery tail-cuff method (BP-2010A; Softron Biotechnology, Beijing, China). All experimental protocols pertaining to laboratory animals were approved by the Ethics Committee of Beijing Sport University and performed in accordance with Chinese animal protection laws and institutional guidelines.

Histological assays

For morphological examination, thoracic aortas were carefully dissected from the connective tissue to avoid mechanical stretching and perfused with 4 % paraformaldehyde for 12 h. The vessels were then rinsed with phosphate buffered solution, dehydrated with ethanol, embedded in paraffin, and cut into 4 µm sections. These tissue sections were stained with hematoxylin and eosin (HE), and images were obtained using an inverted microscope (IX71-F22PH; Olympus, Tokyo, Japan).

Immunohistochemical

Serial thoracic aorta sections (4 µm) were deparaffinized with xylene, rehydrated in graded ethanol, and pretreated with 0.01 M citric acid buffer (pH=6) for 20 min in a thermostatic water bath (99 °C). All materials were pre-incubated with 3 % hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min to ensure endogenous peroxidase inactivation. The sections were

then incubated with 0.3 % TritonX-100 for 15 min, washed 3 times in 0.01 M PBS for 5 min each, and blocked with 5 % normal goat serum in PBS for 20 min. Next, the samples were incubated with anti- α smooth muscle actin (ab5694, Abcam, USA; 1:200), anti-Osteopontin (ab63856, Abcam, USA; 1:100), or anti-Calponin 1 (sc58707, Santa Cruz Biotechnology, USA; 1:200) primary antibodies overnight at 4 °C. Following incubation, the sections were incubated with biotinylated goat anti-rabbit IgG (SA00001-2, Proteintech Group, USA) or anti-mouse IgG (SA00001-1, Proteintech Group, USA) secondary antibodies at a concentration of 1:200 in PBS for 1 h. The sections were then washed 3 times with PBS, visualized with 0.05 % 3,3'-diaminobenzidine (DAB) solution, counterstained with Harris hematoxylin, and imaged on an inverted microscope (IX71-F22PH; Olympus, Tokyo, Japan). Control sections were treated with only secondary antibody.

Western blot analysis

Thoracic aortas were homogenized on ice with lysis buffer, with the supernatant collected after centrifugation. The protein concentration was determined using a Bradford assay, with bovine serum albumin (BSA) used as a standard. Equal amounts of proteins were separated on 10 % SDS-polyacrylamide gels *via* electrophoresis and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with BSA, the membrane was incubated overnight at 4 °C with the primary antibodies anti- α smooth muscle actin (ab5694, Abcam, USA; 1:200), anti-Osteopontin (ab63856, Abcam, USA; 1:100), anti-Calponin 1 (sc58707, Santa Cruz Biotechnology, USA; 1:200), anti-Phospho-Akt (#9271, Cell Signaling Technology, USA; 1:200), anti-Akt (#9272, Cell Signaling Technology, USA; 1:200), anti-Phospho-p44/42 MAPK (Erk1/2) (#9101, Cell Signaling Technology, USA; 1:200), anti-p44/42 MAPK (Erk1/2) (#9102, Cell Signaling Technology, USA; 1:200), anti-Phospho-p38 MAPK (#9215, Cell Signaling Technology, USA; 1:200), anti-p38 MAPK (#9212, Cell Signaling Technology, USA; 1:200), and GAPDH (sc32233, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500). Following the incubation, the membranes were incubated with biotinylated goat anti-rabbit IgG (SA00001-2, Proteintech Group, USA) or anti-mouse IgG (SA00001-1, Proteintech Group, USA) secondary

antibodies at a concentration of 1:10000 in TBST (Tris Buffered Saline with Tween-20) for 1 h, visualized with autoradiography using the ChemiDoc XRS⁺ System (Bio-Rad Laboratories, Hercules, CA, USA), and the band intensities quantified using Image Lab Software (Bio-Rad Laboratories). All values are expressed as a percentage of GAPDH in each lane and then normalized to the value of 1 month.

Statistical analysis

Results are expressed as a mean \pm SEM. Normality tests were performed to determine the presence of a normal distribution, with data log-transformed in the absence of a normal distribution. Two-way analysis of variance (ANOVA) was used to assess the interactive influences of hypertension and aging. *Post hoc* analysis was performed using an independent sample t-test. All analyses were performed using SPSS 19.0 software, with $P < 0.05$ considered significant. Figures were generated using GraphPad Prism 5.

Results

Differences in blood pressure in aging and hypertensive rats

Significant differences were observed between the SHR and WKY groups, with the SHR rats having significantly increased BP (SBP and DBP) and HR when compared with the normal age-matched groups from 3 months and up. When examining the WKY groups ($n=4$), the SBP, DBP and HR tended to be elevated with age. In particular, the SBP and mean arterial pressure showed a significant correlation with both age and hypertension ($P < 0.01$; Fig. 1).

Morphological differences

Thoracic aorta morphological differences among groups were determined by hematoxylin and eosin staining (Fig. 2). Both hypertension and aging significantly contributed to wall thickening ($P < 0.01$), with more pronounced changes seen when hypertension and aging were combined ($P < 0.01$). When comparing SHR-1M and SHR-16M samples, a significant increase in thoracic aortic wall thickness was noted, with this trend was also seen in WKY samples ($P < 0.01$). Moreover, this significant difference occurred as early as 9 months in the SHR groups.

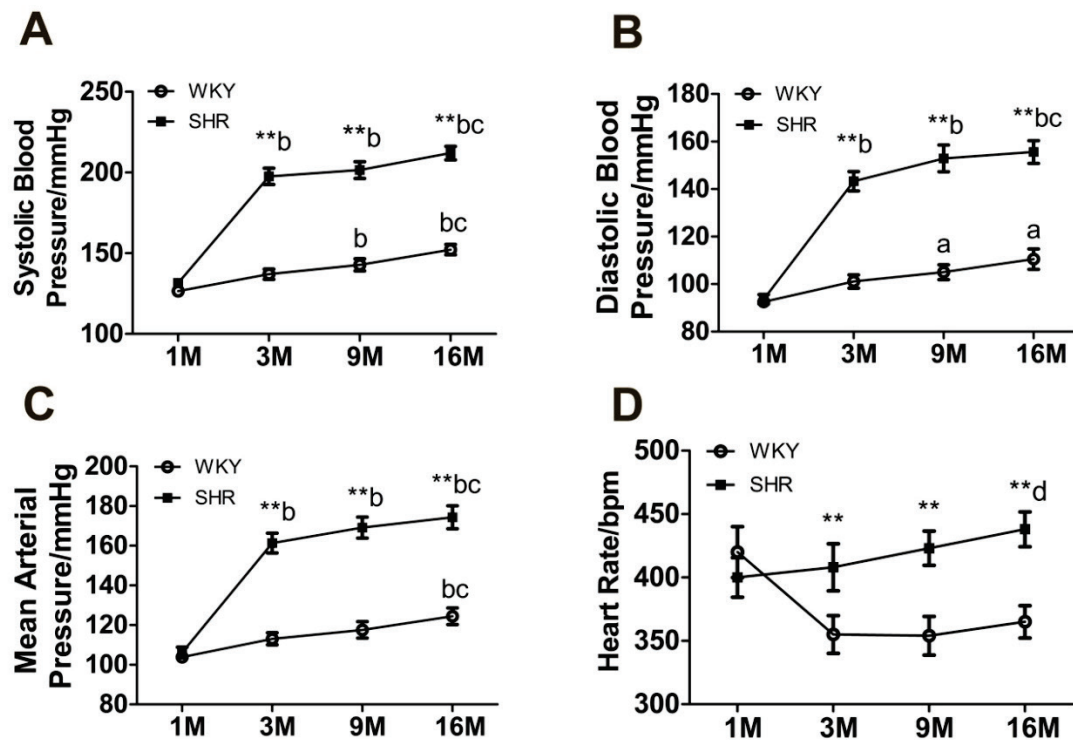


Fig. 1. Graphs showing blood pressure (BP) and heart rate (HR) in the eight groups. Bars indicate SEM ($n=6$). * $P<0.05$ and ** $P<0.01$, sample vs. age-matched WKY; ^a $P<0.05$ and ^b $P<0.01$, sample vs. same strain at 1 month; ^c $P<0.05$ and ^d $P<0.01$, sample vs. same strain at 3 months. (A) Systolic blood pressure (SBP); (B) diastolic blood pressure (DBP); (C) mean arterial pressure (MAP); and (D) heart rate (HR).

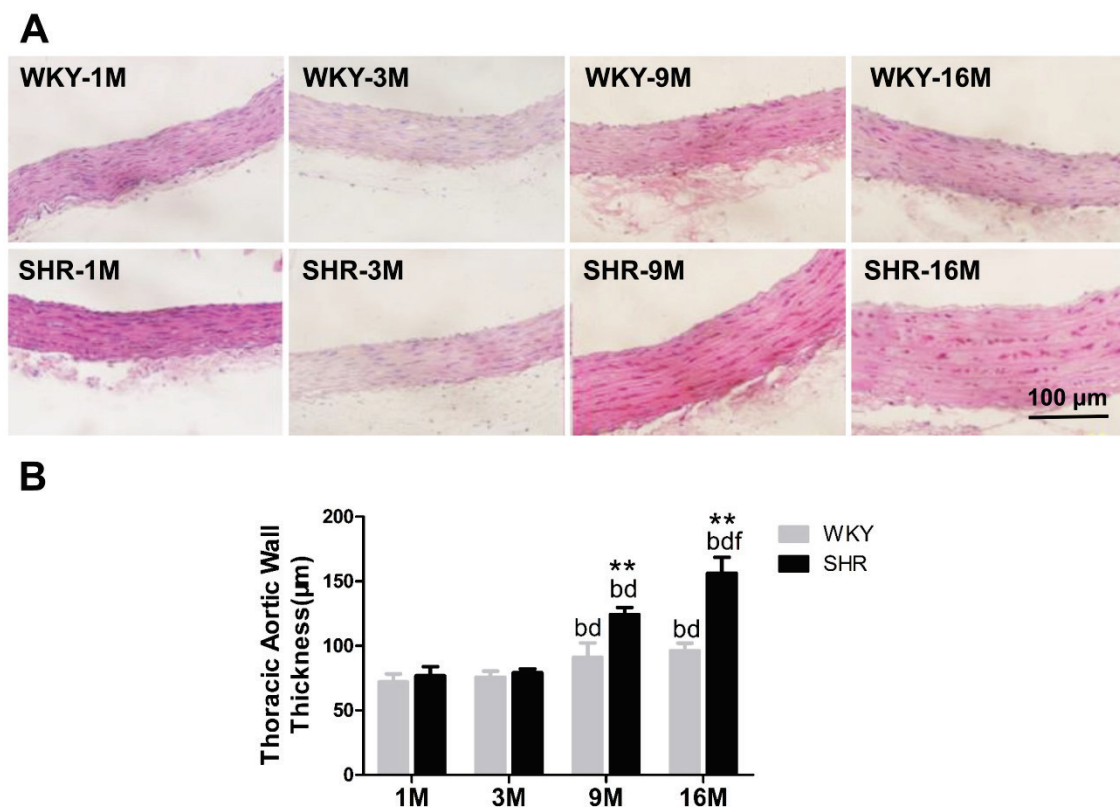


Fig. 2. Histological appearance of the thoracic aorta stained with H&E (hematoxylin and eosin). The results are expressed as a mean \pm SEM ($n=6$). * $P<0.05$ and ** $P<0.01$, sample vs. age-matched WKY; ^a $P<0.05$ and ^b $P<0.01$, sample vs. same strain at 1 month; ^c $P<0.05$ and ^d $P<0.01$, sample vs. same strain at 3 months; ^e $P<0.05$ and ^f $P<0.01$, sample vs. same strain at 9 months. (A) SHR and WKY thoracic aorta of different ages stained with H&E (hematoxylin and eosin). (B) Statistical views of the thoracic aorta wall thickness in eight groups.

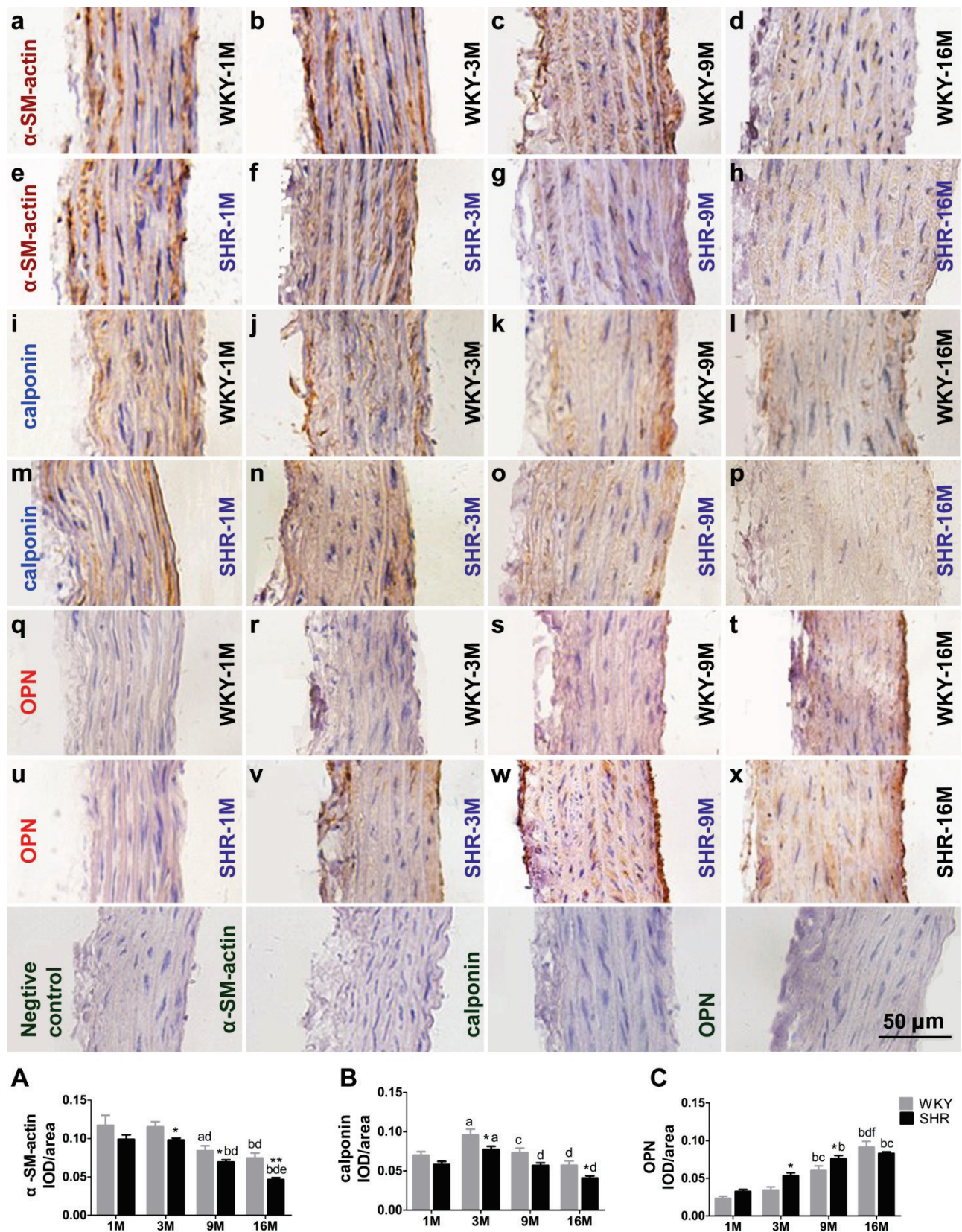


Fig. 3. Immunohistochemistry of the marker proteins in thoracic aorta smooth muscle cells. The results are expressed as a mean \pm SEM (n=6). * P<0.05 and ** P<0.01, sample vs. age-matched WKY; ^a P<0.05 and ^{aa} P<0.01, sample vs. same strain at 1 month; ^b P<0.05 and ^{bb} P<0.01, sample vs. same strain at 3 months; ^c P<0.05 and ^{cc} P<0.01, sample vs. same strain at 9 months.

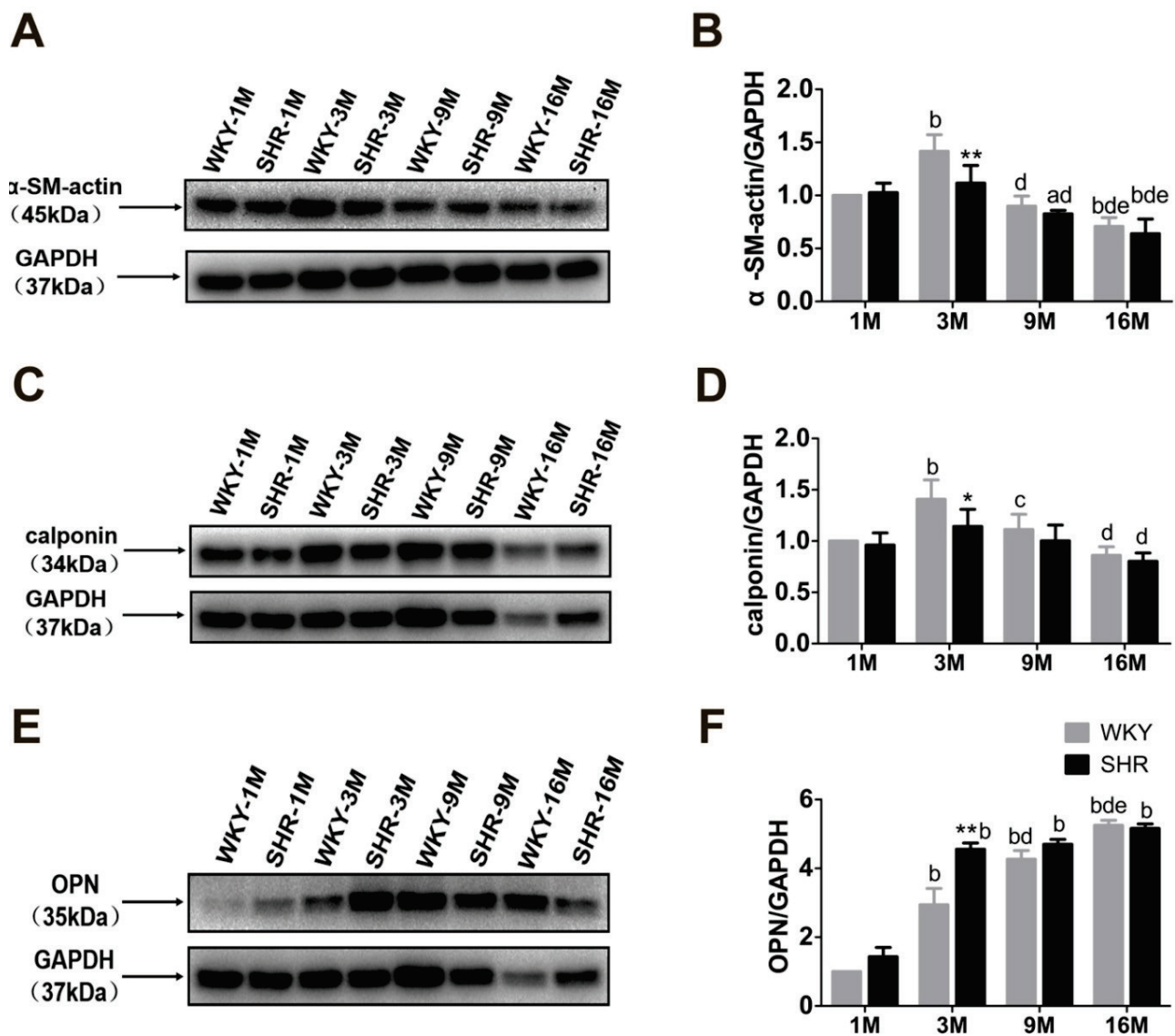


Fig. 4. Western blot of protein markers in thoracic aorta smooth muscle cells. The results are expressed as a mean \pm SEM ($n=6$). * $P<0.05$ and ** $P<0.01$, sample vs. age-matched WKY; ^a $P<0.05$ and ^{aa} <0.01 , sample vs. same strain at 1 month; ^b $P<0.05$ and ^{bb} $P<0.01$, sample vs. same strain at 3 months; ^c $P<0.05$ and ^{cc} $P<0.01$, sample vs. same strain at 9 months. The expression levels of α -SM-actin (A), calponin (C), and OPN (E) were analyzed by Western blot. The quantitative analysis of the expression of α -SM-actin (B), calponin (D), and OPN (F) were performed by Graphpad Prism.

Examination of markers associated with phenotypic switching

Markers associated with phenotypic switching were assessed in both hypertension and aging models *via* immunohistochemistry and Western blot assay. When examining VSMC contractile markers both α -SM-actin and calponin *via* immunohistochemistry and Western blotting, a peak were reached at the age of 3 months, followed by a progressive decrease in both samples. When comparing SHR samples with age-matched WKY samples, these contractile proteins were down-regulated in SHR thoracic aortic smooth muscle cells. When examining hypertension and aging jointly *via*

immunohistochemistry, the expression of the contractile proteins was significant (α -SM-actin: $P<0.01$; calponin: $P<0.01$; Figs 3a-3p, Figs 4A-4D), with α -SM-actin also found to be significant *via* Western blotting ($P<0.01$).

In contrast, the synthetic marker osteopontin (OPN) was increased when examining aging and hypertension jointly (Figs 3q-3x, Figs 4E and 4F). When examining the SHR group, OPN levels were always higher than age-matched WKY samples (3M: $P<0.05$; 9M: $P<0.01$). However, when the age of 16 months was reached, no significant difference between the SHR and WKY groups was noted.

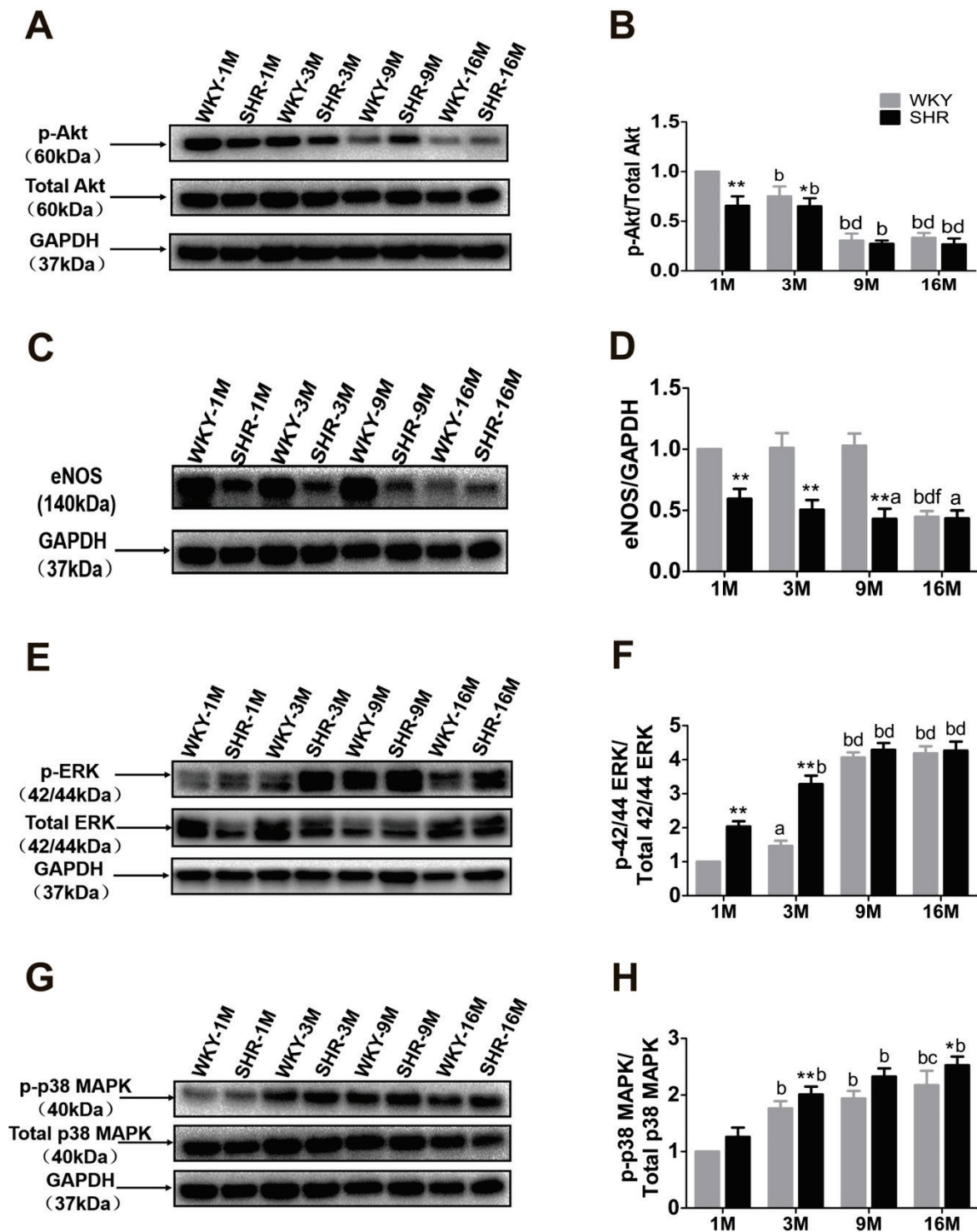


Fig. 5. Western blot of selected signaling pathways proteins in thoracic aorta smooth muscle cells. The results are expressed as a mean \pm SEM (n=6). * P<0.05 and ** P<0.01, sample vs. age-matched WKY; ^aP<0.05 and ^{aa}P<0.01, sample vs. same strain at 1 month; ^bP<0.05 and ^{bb}P<0.01, sample vs. same strain at 3 months; ^cP<0.05 and ^{cc}P<0.01, sample vs. same strain at 9 months. The phosphorylation of Akt (A) and the protein expression of eNOS (C) were reduced accompanied by the aging and hypertension. However, the phosphorylation of ERK (E) and p38 MAPK (G) were increased with aging and hypertension. The quantitative analysis of the expression of these proteins (B), (D), (F) and (H), respectively.

The expression level of signaling pathways proteins in VSMCs in hypertension and aging rats

To further analyze signal transduction relating to the thoracic aortic VSMC phenotype, AKT/eNOS

signaling was examined *via* Western blotting. These results showed that p-AKT and eNOS levels decreased with increasing age in both the WKY and SHR groups (Figs 5A-5D). In the SHR group, p-AKT was

significantly down-regulated at the ages of 1 ($P < 0.01$) and 3 ($P < 0.05$) months compared with the age-matched WKY samples, but with no significant difference noted at the ages of 9 and 16 months. When examining eNOS expression, the SHR group showed a significant down-regulation at the ages of 1, 3, and 9 month ($P < 0.01$), but no significant difference was noted at the age of 16 months.

MAPK pathway signaling was also examined, with ERK1/2 and p38 MAPK specifically probed. These proteins were found to have significantly higher expression in the SHR group relative to age-matched WKY group samples (Figs 5E-5H). When examining hypertension and aging jointly, both p-ERK1/2 and p-p38 MAPK were significantly increased. Moreover, the interactions between hypertension and aging were significant for p-ERK1/2 ($P < 0.01$), but not for p-p38 MAPK.

Discussion

In this study, hypertension and aging were examined jointly to gain insight into the mechanisms of phenotypic switching in thoracic aorta smooth muscle cells. Aging is associated with well-defined phenotypic changes that make the cardiovascular system prone to disease even in the absence of traditional risk factors (e.g. hypertension, stroke, or coronary heart disease). Similarly, hypertension strongly contributes to cardiovascular disease *via* phenotypic switching in VSMCs. Herein, hypertension and aging were found to accelerate phenotypic switching progression, with a more significant progression seen when these factors were combined. During hypertension and aging, the contractile protein markers expression gradually tapered. However, when examining a synthetic (proliferative) type marker, the opposite trend was seen. When examining Akt/eNOS and MAPK signaling, the Akt/eNOS pathway was decreased accompanying hypertension and aging, while ERK and p38 MAPK expression was down-regulated. These findings would suggest that the VSMC phenotype is determined by a balance between the effects of the Akt and MAPK pathways.

Hypertension and age are inseparable in the process of vascular remodeling, with both contributing to cardiovascular disease (Dan *et al.* 2015). Progressive changes in the structure and function of arteries occurs throughout life and includes diffuse intimal thickening and medial degeneration, increased stiffening, and

reduced distensibility of large arteries (Lakatta 2015). Arterial morphology is regulated by shear stress from the pulsatile blood pressure and flow (Elvebak *et al.* 2010). Arterial hypertension accelerates the progression of atherosclerosis, a pathologic chronic inflammatory process of large and middle-sized arteries that shows an age-dependent progression (Barton 2010). In this study, both hypertension and aging increased rat BP, both SBP and DBP. Furthermore, thoracic aorta wall thickness was increased in both the aging and hypertension models. These results are consistent with data from both animal and human studies that indicate that superimposing aging and hypertension affects BP and vessel morphology (Kotsis *et al.* 2011, Sehgel *et al.* 2015). Herein, morphological examination revealed an enhanced medial thickness in the presence of increased age and BP.

While phenotypic switching has been implicated with VSMC dysfunction associated with hypertension, the exact mechanisms and associated signal transduction remains unclear (Kawahara *et al.* 2005, Laplante *et al.* 2003, Ushio *et al.* 1999). There are two phenotypic types in VSMC, contractile (differentiated) and synthetic (proliferative or de-differentiated). VSMCs change from a contractile state to a synthetic one during vascular impairment, hypertension, or atherosclerosis (Ross 1993). These two phenotypes can be distinguish using specific markers, with the contractile phenotype determined using α -SM-actin, h1-calponin, SM22 α , or SM-MHC, while the synthetic phenotype is determined with OPN (Baria *et al.* 1986, Christen *et al.* 1999, Han *et al.* 2009, Jimenez *et al.* 2012). In recent studies, OPN has been found to play an important role in the process of myocardial reconstruction, has been found to be up-regulated in cardiovascular disease models, and has a close association with inflammation and hypertension (Rosenberg *et al.* 2008, Stepien *et al.* 2011, Rosenberg *et al.* 2012, Gao *et al.* 2012). In the present study, SHR samples exhibited a decrease α -SM-actin and calponin expression accompanied by increased OPN expression when compared with age-matched WKY samples. Additionally, aging WKY samples showed significantly lower α -SM-actin and calponin expression, while OPN expression remained up-regulation (Figs 3 and 4). When superimposing aging and hypertension, the contractile markers were down-regulated, while the synthetic marker was up-regulated.

The mechanisms regulating VSMC phenotypic switching and the critical signal transduction affecting this phenotype remain controversial. While the

PI3K/Akt/eNOS and Ras/Raf/MAPK pathways have been implicated in many cellular activities ranging from gene expression to mitosis, movement, proliferation, migration, and apoptosis (Zhu *et al.* 2015, Yang *et al.* 2013, Ouyang *et al.* 2014, Xue *et al.* 2016, Chen *et al.* 2015, Li H. *et al.* 2014), their potential roles in phenotypic switching remains unclear. In a study, the maintenance of a contractile phenotype in VSMC was shown to depend on the Akt pathway, whereas the coordinate activation of the ERK and p38 MAPK pathways induced proliferation, thus indicating that VSMC phenotype is determined by a balance between these pathways (Umemoto *et al.* 2006). However, how hypertension and age jointly impact the regulation of the VSMC phenotype is still unclear. Thus, this study examined these two factors jointly while monitoring the Akt/eNOS and ERK/MAPK pathways to better characterize phenotypic switching. In the SHR group, p-Akt and eNOS expression showed a downward trend when compared with age-match WKY samples. In the normal group, p-Akt and eNOS expression decreased with increasing age. Thus these findings suggest that age and hypertension when acting jointly accelerate the process of phenotypic transformation through reduced AKT signaling. When examining the ERK and p38 MAPK pathways, p-ERK and p-p38 MAPK expression was up-regulated in the SHR groups relative to the age-matched WKY group, with the same trend seen when examining just the effect of aging. Interestingly, when examining hypertension and aging jointly, a more significant change in expression was noted.

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In general, this study demonstrates that hypertension and aging exert a compounded influence on the vascular system, especially relating to BP and vascular morphology. Furthermore, the Akt and MAPK pathways serve critical roles in vascular phenotypic switching during hypertension and aging. Importantly, the impacts of hypertension and aging are compounded when considered jointly and result in a reduction in Akt pathway signaling, while MAPK pathway signaling is promoted.

While previous work has focused on VSMC phenotypic modulation, this study demonstrates that the intrinsic mechanical properties of VSMCs are associated with vascular remodeling in hypertension and aging. Furthermore, the interplay between hypertension and aging may be responsible for the acceleration of vascular remodeling. In the present study, only a small number of signaling pathways pertaining to VSMC phenotype switching were examined, but other pathways relating to phenotypic modulation will be assessed *in vivo* and *in vitro* in future studies.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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