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Acupuncture Research

Electroacupuncture Attenuated Phenotype Transformation of Vascular Smooth Muscle Cells via PI3K/Akt and MAPK Signaling Pathways in Spontaneous Hypertensive Rats*

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ABSTRACT **Objective**: To investigate whether the antihypertensive mechanism of electroacupuncture (EA) is associated with attenuating phenotype transformation of vascular smooth muscle cells (VSMCs) via phosphoinositide3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) signaling pathways. **Methods**: Eight Wistar-ktoyo (WKY) rats were set as normal blood pressure group (normal group). A total of 32 spontaneous hypertensive rats (SHRs) were randomly divided into 4 groups using random number tables: a model group, an EA group, an EA+PI3K antagonist group (EA+P group), and an EA+p38 MAPK agonist+extracellular signal-regulated kinase (ERK) agonist group (EA+M group) (n=8/group). SHRs in EA group, EA+P group and EA+M group received EA treatment 5 sessions per week for continuous 4 weeks, while rats in the normal and model groups were bundled in same condition. The systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) of each rat was measured at 0 week and the 4th week. After 4-week intervention, thoracic aorta was collected for hematoxylin-eosin (HE) staining, immunohistochemistry [the contractile markers α-smooth muscle actin (α-SMA) and calponin and the synthetic marker osteopontin (OPN)] and Western blot [α-SMA, calponin, OPN, PI3K, phosphorylated-Akt (p-Akt), Akt, p-p42/44 ERK, total p42/44 ERK, p-p38 MAPK and total p38 MAPK]. **Results**: EA significantly reduced SBP, DBP and MAP (P<0.01). HE staining showed that the wall thickness of thoracic aorta in EA group was significantly decreased (P<0.01). From results of immunohistochemistry and Western blot, EA increased the expression of α-SMA and calponin, and decreased the expression of OPN (P<0.01). In addition, the expression of PI3K and p-Akt increased (P<0.01), while the expression of p-p42/44 ERK and p-p38 MAPK decreased in EA group (P<0.01). However, these effects were reversed by PI3K antagonist, p38 MAPK agonist and ERK agonist. **Conclusions**: EA was an effective treatment for BP management. The antihypertensive effect of EA may be related with inhibition of phenotypic transformation of VSMCs, in which the activation of PI3K/Akt and the repression of MAPK pathway were involved.

KEYWORDS electroacupuncture, hypertension, vascular smooth muscle cells, PI3K/Akt signaling pathway, MAPK signaling pathway

Hypertension has become the leading risk-factor for cardiovascular disease worldwide.[1] The results from the China Hypertension Survey (2012–2015) showed that 23.2% of the Chinese adults had hypertension, and 41.3% had pre-hypertension according to the Chinese guideline.[2] Besides, owing to aging population and people's unhealthy lifestyle, the prevalence of hypertension is steeply increasing.[3] However, the control rate of hypertension is quite low. A multinational population study indicated that only 32.5% of hypertensive patients were under management.[4]

Electroacupuncture (EA), a classical method of Chinese medicine, has been recognized as an effective treatment for high blood pressure (BP).[5] Li, et al[6] found that EA treatment was superior to sham acupoint

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group in lowering BP. Our previous clinical study[7] also found that compared with sham acupuncture and waiting-list groups, EA could significantly decrease systolic blood pressure (SBP). However, the mechanism of EA for hypertension is still unclear.

Vascular remodeling was considered as an important pathophysiological basis for the occurrence and development of hypertension.[8-10] Vascular smooth muscle cells (VSMCs), major cellular components of blood vessel, preserve evident phenotypic plasticity[11] and are able to undergo a reversible phenotypic transformation (from contractile to synthetic phenotype) in response to various stimuli.[12] Proteins maintaining the contractile phenotype include α-smooth muscle actin (α-SMA), calponin, smooth muscle myosin heavy chain protein (SM-MHC), etc., while proteins maintaining the synthetic phenotype include osteopontin (OPN) and epiregulin. Numerous studies showed that phenotype transformation of VSMCs was an important cause of vascular remodeling.[13,14] In addition, several studies indicated that phenotype transformation of VSMCs could be regulated by phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) and the mitogen-activated protein kinases (MAPK) pathways.[15-18]

Our previous animal experiments found that the antihypertensive effect of EA was related with PI3K/Akt pathway and MAPK signaling pathway in spontaneous hypertensive rats (SHRs).[19,20] Based on the above evidence, we hypothesized that EA could exert antihypertensive effect by alleviating phenotype transformation of VSMCs via PI3K/Akt and MAPK signaling pathways. Therefore, with the aim to explore the mechanism of EA, we selected SHRs as hypertensive model, and observed the effect of EA on wall thickness of thoracic aorta, the expressions of α-SMA, calponin and OPN, and the expressions of PI3K/Akt and MAPK pathways with hematoxylin-eosin (HE) staining, immunohistochemistry (IHC) and Western blot analysis.

METHODS

Reagents and Consumables

Stainless acupuncture needles prepared by Hua-Tuo Medical Appliance Factory, Suzhou, China and EA apparatus (HANS-200) was from Nanjing, China. The primary reagents and consumables are listed in Appendix 1. The primary and secondary antibodies used in this study are listed in Appendix 2.

Animals and Groups

Thirty-two male 2-month-old SHRs and 8 Wistar-ktoyo (WKY) rats weighing 200–250 g were purchased from Chengdu Dashuo Experimental Animal Co., Ltd., China (specific pathofen-free grade, certificate No. SCXK-2019-030). Adequate food and tap water were freely available to all animals. The rats were placed in an environment with a controlled temperature (22 ℃–26 ℃) and a relative humidity of 50%–70%, and the light/dark cycle lasted for 12 h. All experimental personnel participated in experiments under the supervision and guidance of the Laboratory Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine. All procedures were in compliance with the Statute on the Administration of Laboratory Animal approved by China's Council (November 1988).

After a week of adaptive feeding, 8 WKY rats were set as normal BP group (normal group). Thirty-two SHRs were randomly assigned to 4 groups using random number tables: a model group, an EA group, an EA + PI3K inhibitor group (EA + P group), an EA + p38 MAPK agonist + ERK agonist group (EA + M group), 8 in each group. The flow chart of this experiment was presented as Figure 1.

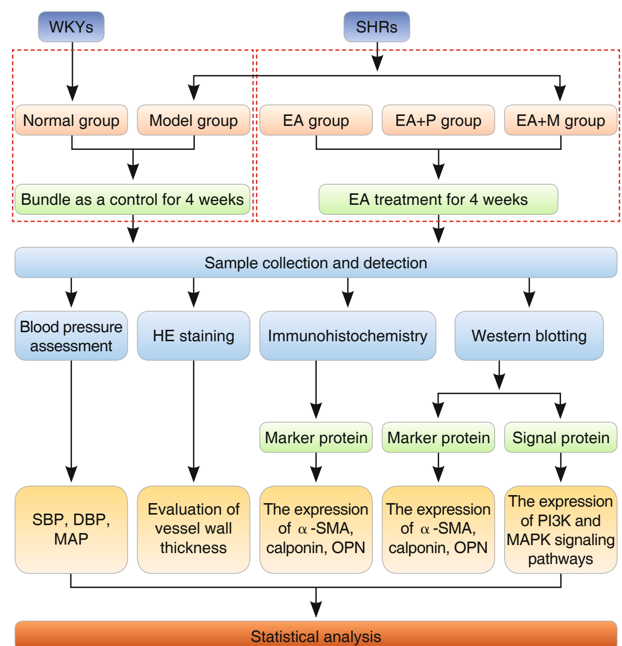

Figure 1. Flow Chart of this Experiment

Notes: EA: electroacupuncture; EA + P group: EA + PI3K antagonist group; EA + M group: EA + p38 MAPK agonist + ERK agonist group; SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial blood pressure; α-SMA: α-smooth muscle actin; OPN: osteopontin

Drug Administration

LY294002, an antagonist of PI3K, 0.3 mg/kg per day,[21] was purchased from TargetMol, USA; anisomycin, an agonist of p38MAPK, 0.1 mg/kg daily,[22,23] from TargetMol, USA; and EGF, an ERK activator, 250 μg/kg daily[24] from Peprotech, USA. All drugs were dissolved in 2% dimethyl sulfoxide, and aliquots were stored at –20 ℃ until use. Aliquots of these drugs were intraperitoneally injected in SHRs of EA+P and EA + M groups, 1 h before each EA treatment.

EA Treatment

SHRs in EA, EA + P and EA + M groups received EA treatment once a day. Stainless acupuncture needles (0.25 mm × 25 mm) were inserted into Renying (ST 9), Quchi (LI 11), Zusanli (ST 36), and Fenglong (ST 40) on both sides of rats' bodies at a depth of 5 mm. Then EA apparatus was connected to needles to generate stimulation. The stimulation parameters were set as follows:[16,25] disperse wave of 2 Hz; 20 min per session. Rats in the normal and model groups were bundled as blank control procedure for 20 min per day without any treatment. The EA treatment and blank procedure were delivered 5 times per week for continuous 4 weeks. During EA procedure, we tied the rats on boards with packaging tape (Figure 2).

Figure 2. The Method of Tiding Rats during EA Procedure

Collection of Tissue Samples

The rats were anesthetized with 10% chloral hydrate and euthanized after 4-week treatment. Then, the thoracic aorta was separated and washed by phosphate buffered solution (PBS) twice. A part of the thoracic aorta tissues was fixed in 4% paraformaldehyde for 24 h for HE staining and IHC. The remaining thoracic aorta tissues were preserved by liquid nitrogen in the frozen pipes for the measurement of expression of relevant proteins.

Measurement of BP

BP was measured using tail-cuff system (BP2000, Visitech System, USA). The rats were kept in dark and warmly restrained, then BP was measured from caudal artery. The SBP, diastolic blood pressure (DBP) and mean arterial pressure (MAP) of each rat were measured at baseline and the 4th week.

HE Staining

Thoracic aorta was fixed in 4% paraformaldehyde for 24 h, paraffin-embedded and sectioned. The sections were stained by HE for 10 min, differentiated by 1% hydrochloric alcohol for 10 s, then treated by 2% sodium bicarbonate for 10 s; disseminated in eosin for 3 min, dehydrated in ascending series of ethanol, cleared in xylene, mounted and captured. Meanwhile, the thickness of vessel wall was measured. The images were obtained using a light microscope (BA400DIGITAL Nikon, Tokyo, Japan). The wall thickness of thoracic aorta at 3 random points of each sample was measured with Image J V1.8.0 software (NIH, Bethesda, MD, USA).

IHC

Thoracic aorta tissues were fixed with 4% paraformaldehyde and routinely processed for embedding into paraffin blocks. Blocks were cut using a microtome into 4-μm-thick sections. Sections were deparaffinized in xylene and rehydrated through a graded ethanol series. In order to retrieve antigen activity, sections were incubated with citric acid buffer (pH 6.0) for 30 min at 100 ℃, then endogenous peroxidase activity was blocked by incubating section for 30 min in 3% H_2O_2. The sections were then blocked for 60 min with 5% non-fat milk in tris buffer solution with tween (TBST) and subsequently incubated with primary antibodies against OPN (1:200), α-SMA (1:500) and calponin 1 (1:200) at 4 ℃ overnight. After washing in PBS buffer, the sections were incubated in a 37 ℃ incubator for 0.5 h. Besides, PBS buffer replaced primary antibodies as negative control group. The images were obtained using a light microscope (Nikon, Tokyo, Japan). Positive area from 3 random points of each sample was calculated using Image J software.

Western Blot

Thoracic aorta homogenate was prepared and

total protein content of thoracic aorta was evaluated according to the instruction of bicinchoninic acid (BCA) kit (P0011, Beyotime Biotechnology Co., Ltd., Shanghai, China). Proteins were mixed with loading buffer, boiled at 95 ℃ for 10 min, and speared with 10% polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane, then sealed with 5% non-fat milk in TBST for 1 h, added with primary antibodies α-SMA (1:2000), calponin 1 (1:500), OPN (1:500), PI3K (1:500), Akt (1:500), p-Akt (1:500), p42/44ERK (1:500), p-p42/44ERK (1:500), Tubulin (1:5000), GAPDH (1:5000), p38MAPK (1:500) and p-p38MAPK (1:500), incubated at 4 ℃ for one night, rinsed by TBST, 3 times/5 min and incubated by relative secondary antibody for 1 h. Then, the membrane was added with enhanced chemiluminescent regent (ECL), exposed in darkroom, and GAPDH or Tubulin were used as internal reference. After photography analyzed by Vision Works system, the target bands were conducted with grey value analysis by Image J software.

Statistical Analysis

All data were analyzed by SPSS 25.0 software (SPSS, Inc., Chicago, IL, USA). The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), comparisons among multiple groups were assessed by one-way analysis of variance (ANOVA). Paired *t* test was used to compare the samples within the group. *P*-values <0.05 were considered statistically significant.

RESULTS

Effects of EA on BP

At baseline, SHRs showed significantly higher SBP, DBP and MAP than WKYs (*P*<0.01). After 4-week intervention, rats in EA group showed more decreased SBP, DBP and MAP than rats in the model group (*P*<0.01). Compared to EA group, higher SBP, DBP and MAP were observed in EA + P group and EA + M group (all *P*<0.05, Table 1).

EA Attenuated Aortic Remodeling in SHRs

Morphological data indicated that the thickness of thoracic aortas was significantly increased in the model group versus normal group (*P*<0.01). Compared with the model group, the wall thickness of thoracic aorta in EA group was decreased (*P*<0.01). Compared with EA group, the wall thickness of thoracic aorta in EA + P and EA + M group was increased (*P*<0.01, Figure 3).

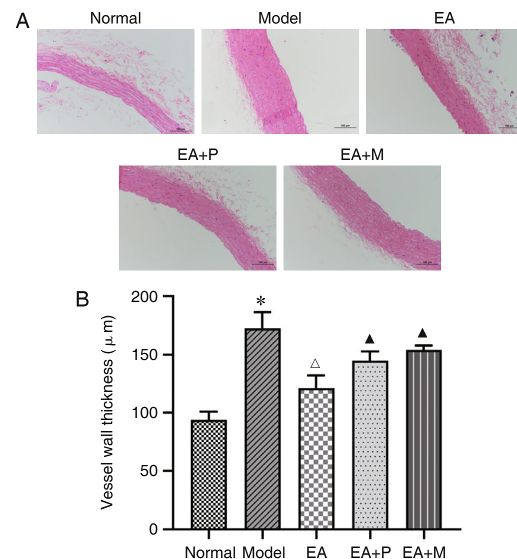

Figure 3. Effect of EA Treatment on Wall Thickness of Thoracic Aortas

Notes: A: HE staining of thoracic aorta in each group (*n*=4/group, scale=100 μm, 200×); B: Relevant quantitative analysis of the wall thickness of thoracic aorta from each group; **P*<0.01 *vs*. normal group; △*P*<0.01 *vs*. model group; ▲*P*<0.01 *vs*. EA group

EA Regulated VSMCs Marker Protein Expression

According to the results of IHC (Figures 4A–4D), compared with the normal group, the expression of the contractile markers (α-SMA and calponin) signifficantly down-regulated in model group (*P*<0.01), and the expression of the synthetic marker OPN up-regulated in model group (*P*<0.01), while EA increased the expression of α-SMA and calponin, decreased the expression level of OPN (*P*<0.01). Compared with EA group, the expression of α-SMA and calponin

Table 1. Blood Pressure of Each Group at 0 week and 4th week ($\bar{x} \pm s$, mm Hg)

Group	*n*	SBP		DBP		MAP	
		0-week	4-week	0-week	4-week	0-week	4-week
Normal	8	131.12±4.34	132.50±5.08	101.16±5.63	102.03±8.91	111.15±3.68	112.19±5.30
Model	8	201.22±12.43*	208.72±12.51*○○	140.35±6.91*	147.13±11.30*○	160.64±8.52*	167.66±10.65*○
EA	8	201.89±10.67	186.20±8.25△△○○	141.10±6.01	133.23±5.20△△○○	161.36±7.07	150.89±5.36△△○○
EA+P	8	203.20±12.53	198.41±11.54▲	142.11±5.96	140.01±8.17▲	162.64±8.17	159.48±7.31▲
EA+M	8	202.95±12.89	199.11±9.44▲	143.14±4.33	141.27±5.71▲	163.08±7.01	160.55±6.54▲

Notes: **P*<0.01 *vs*. normal group; △△*P*<0.01 *vs*. model group; ▲*P*<0.05, ▲▲*P*<0.01 *vs*. EA group; ○*P*<0.01, ○○*P*<0.01 *vs*. 0 week

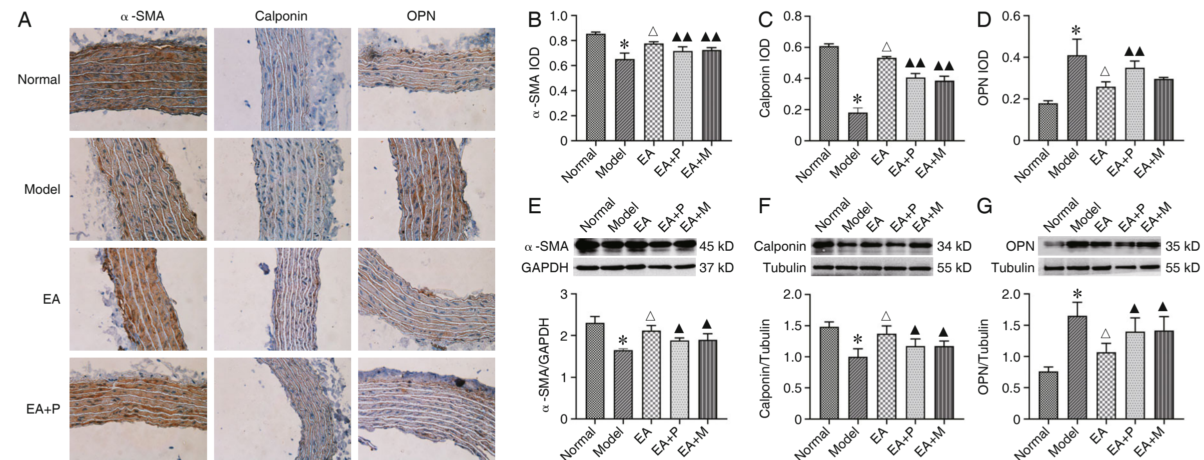

Figure 4. Effect of EA on VSMCs Marker Protein Expression

Notes: A: Immunohistochemical map of α-smooth muscle actin (α-SMA), calponin and the synthetic marker osteopontin (OPN) in each group (*n*=4/group, scale=100 μm, 200×). The analysis results of α-SMA, calponin and OPN are shown in B, C and D, respectively. Marker expression levels are shown in E, F, G with Western blot **P*<0.01 *vs.* normal group; △*P*<0.01 *vs.* model group; ▲*P*<0.05, ▲▲*P*<0.01 *vs.* EA group

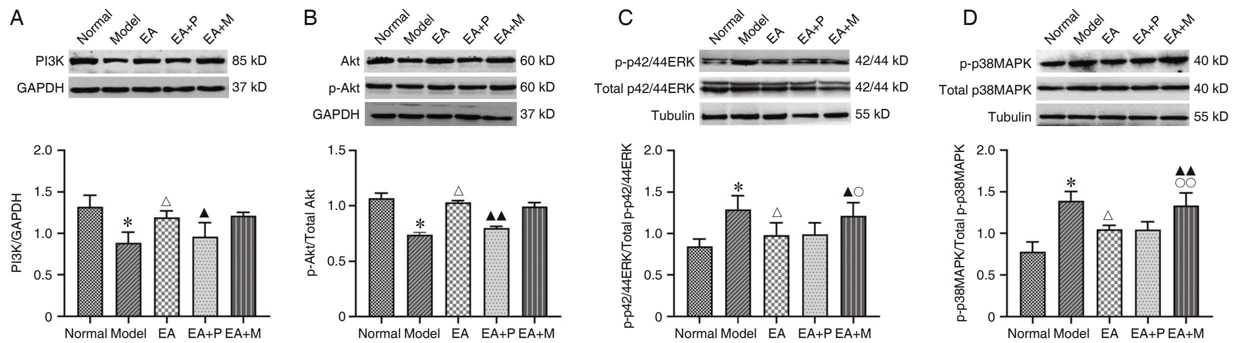

Figure 5. Effect of EA on PI3K/Akt and MAPK Pathways

Notes: Marker expression levels are measured with Western blot. **P*<0.01 *vs.* normal group; △*P*<0.01 *vs.* model group; ▲*P*<0.05, ▲▲*P*<0.01 *vs.* EA group; ○*P*<0.05, ○○*P*<0.01 *vs.* EA + P group; *n*=4/group

reduced in EA+P and EA+M groups (*P*<0.01), and the expression of OPN increased in EA+P group (*P*<0.01). Western blot detections got the same results, except for the expression of OPN in EA+M group (Figures 4E–4G). Compared with EA group, the expression of OPN increased in EA+M group (*P*<0.05).

EA Activated PI3K/Akt Pathway and Repressed MAPK Pathway in SHRs

According to the results of Western blot (Figure 5), compared with the normal group, the expression of PI3K and p-Akt decreased (*P*<0.01) while the expression of p-p42/44ERK and p-p38MAPK increased (*P*<0.01) in the model group. Compared with the model group, the expression of PI3K and p-Akt increased (*P*<0.01), while the expression of p-p42/44ERK and p-p38MAPK decreased in EA group (*P*<0.01). Compared with EA group, the expression of PI3K and p-Akt decreased in EA+P group (*P*<0.05 or *P*<0.01); the expression of p-p42/44ERK and p-p38MAPK up-regulated, but no significant difference was found (*P*>0.05); and in EA+M group, the expression of p-p38MAPK and p-p42/44ERK was enhanced (*P*<0.01 or *P*<0.05), while the expression of PI3K and p-Akt showed no significant difference (*P*>0.05).

DISCUSSION

Hypertension is a leading cause for cardiovascular disease and all-cause mortality in the world,[26,27] which is charactered as high prevalence and low control rate, and remains as a major public health challenge. EA is commonly used as a treatment and prevention method for hypertension.[28] However, the underlying mechanism of EA on hypertension was not fully

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understood. Previous studies have indicated that phenotype transformation of VSMCs modulated by PI3K/Akt pathway and MAPK pathway was crucial for the occurrence of hypertension.[29,30]

We adopted SHR as rodent model, a common and natural animal model of hypertension,[31] to explore the underlying mechanisms. Our previous data mining study demonstrated that ST 9, LI 11, ST 36, and ST 40 were the most frequently used acupoints in treating hypertension.[32] And previous clinical study[7] has also confirmed the anti-hypertensive effect in hypertension patients. Li, et al[16] and Zheng, et al[25] found that parameters of EA treatment (disperse wave of 2 Hz; 20 min per day for 4 consecutive weeks) has better anti-hypertensive effect in SHRs. The findings of our study are summarized as followed (Figure 6): (1) EA was capable to lower BP and reverse the vascular morphological changes in SHRs; (2) EA treatment significantly increased the expression of α-SMA and calponin, and decreased the expression of OPN in SHRs; (3) EA could regulate VSMCs phenotype switching by activating PI3K/Akt and repressing MAPK signal pathways in SHRs.

Firstly, our results showed that EA treatment had anti-hypertensive effect in SHRs. Compared to model group, the SBP, DBP and MAP of EA group decreased. The results were consistent with current evidence. Li, et al[33] reported that 8-week EA treatment could decrease peak and average SBP and DBP in hypertensive patients. Hwang, et al[34] reported that EA could lower more SBP and DBP than non-acupuncture point and no treatment control group in SHRs. A network meta-analysis also indicated that acupuncture was an effective method for treating hypertension.[35]

Secondly, we found that EA treatment significantly attenuated the phenotypic transformation of VSMCs through increasing the expression of α-SMA and calponin, and decreasing the expression of OPN in SHRs. As we known, BP is tightly regulated by blood vessel, which is comprised of VSMCs.[36] VSMCs preserve evident phenotypic plasticity[11] and could undergo a reversible phenotypic transformation in response to various stimuli.[12] The phenotype transformation of VSMCs refers to a change of VSMCs from contraction phenotype (differentiated state) to synthesis phenotype (dedifferentiated state).[37] In last decades, researchers have found that a host of proteins are crucial for the differentiated function of the VSMCs including α-SMA, SM-MHC, etc.[38] In addition, calponin, as part of the cytoskeleton, has been indicated that it is involved in regulation of contraction.[39]

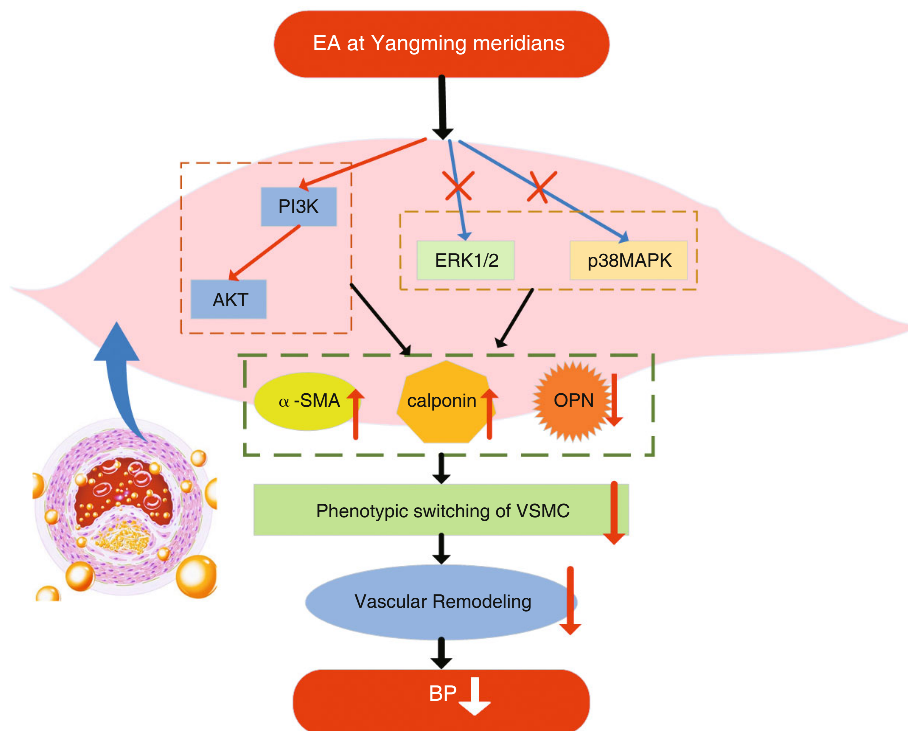

Figure 6. Antihypertensive Effect of EA by Inhibiting Phenotypic Switching via Regulation of PI3K/Akt and MAPK Signaling Pathways

OPN is considered as a protein marker showing VSMCs transformed from contraction phenotype to synthesis phenotype. What's more, it has been confirmed that VSMCs phenotype transformation is involved in the whole process of vascular remodeling in SHRs.[40,41] Vasculature remodeling is an active process of structural change and resulted in the increase of vessel wall thickness and elevation of BP.[42] A clinical study discovered that 94.3% of 179 hypertensive patients had vascular remodeling.[43] Therefore, inhibition of phenotype switching in VSMCs may be a new treatment strategy for hypertension.[44] Our results were consistent with other studies. Zhang, et al[45] found that compared with WKY rats, SHRs showed obvious vascular remodeling, characterized as increased lumen diameter in aorta, thickened intima-media, disorganized VSMCs and elastic fibers in carotid artery, thickened vessel wall with hyaline change and stenotic or occluded lumen in mesenteric small artery and intracranial arterioles. Hao, et al[46] also reported that the wall thickness and ratio of thickness to diameter of vascular wall were significantly decreased in SHRs after EA treatment.

Thirdly, we also found that EA attenuated phenotype transformation via activation of PI3K/Akt signaling pathway and repression of MAPK pathway. Consistent with our previous experiment,[19,20] EA at ST 9, LI 11, ST 36, and ST 40 could activate PI3K/Akt signaling pathway and repress MAPK pathway in SHRs. Fu, et al and Dong, et al.[47,48] also reported that EA at LI 11 and ST 36 was able to decrease the p38MAPK mRNA in prehypertension rats. In addition, another study indicated that EA could activate PI3K/Akt signaling pathway in SHRs.[49] Currently, limited studies investigated the mechanism of EA for hypertension based on the phenotype transformation of VSMCs. *In vitro*, it has been demonstrated that the activation of PI3K/Akt signaling pathway helped to maintain the contractile phenotype of VSMCs, while the activation of MAPK signaling pathway helped to maintain the synthetic phenotype of VSMCs.[21,50,51] Lu, et al[14] found that the activation of PI3K/Akt pathway induced by nesfatin-1 was involved in the phenotype switching of VSMCs in hypertensive vascular remodeling. Zhang, et al[52] confirmed that hypertension decreased the contractile phenotype markers (α-SMA and calponin), while elevated the synthetic phenotype maker (OPN), which was accompanied with the inhibition of Akt signaling pathway and the activation of MAPK signaling pathway. Another study showed that activation of PI3K/Akt pathway elicited by exercise played a positive role in increasing the expression of α-SMA and calponin, and decreasing the expression of OPN, while MAPK pathway had opposite effect.[51]

However, the present study has certain limitations. Firstly, we didn't set sham acupoint as a comparison. The specific therapeutic effect of these acupoints may not be fully confirmed in this study. Secondly, we didn't perform arteria BP monitoring. The data of BP fluctuations within 24 h were not observed. Thirdly, only the vessel wall thickness was measured as the indicator for vascular remodeling, the ratio of wall thickness to inner diameter and the ratio of wall cross-sectional area to luminal cross-sectional area could be detected in subsequent studies. In addition, we adopt intraperitoneal injection of antagonist and agonist. Regarding the metabolism and absorption of antagonist and agonist, gene silencing or knock out should be used in the future.

EA is an effective treatment for BP management. The anti-hypertensive effect of EA may be related with inhibition of phenotypic switching of VSMCs, in which the activation of PI3K/Akt and repression of MAPK pathway were involved.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

The experiment was carried out by all the authors. Chen XY, Yang LP and Zheng YL drafted the manuscript. Li YX and Zhong DL analyzed the data. Jin RJ and Li J revised the manuscript.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Electronic Supplementary Material: Supplementary materials (Appendixes) are available in the online version of this article at https://doi.org/10.1007/s11655-021-2883-y

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