

Original Article

Hypertensive venous changes and Eph-B4/caveolin-1 pathways: an exploratory study

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Abstract: Hypertension is a major risk factor for cerebral vascular disease (CVD). Previous studies have only focused on cerebral artery disease, failing to attach importance to the role of the cerebral venous system in CVD. The aim of this study was to investigate the structural change of jugular veins and to explore whether Eph-B4/caveolin-1 (Cav-1) pathways plays a role in jugular venous changes caused by arterial hypertension. A stroke-prone renovascular hypertensive rat (RHRSP) model was established. Expression of MMP-9, EphB4, Cav-1, and p-Cav-1 was detected through Western blot, PCR, immunohistochemistry, or immunofluorescence. Jugular veins in the RHRSP group were thicker than those in the sham group, as were the intima of the jugular veins, mainly composed of type I collagen. Compared to the sham group, expression of Eph-B4 was significantly decreased and levels of Caveolin-1 (Cav-1) and phosphorylated Caveolin-1 (p-Cav-1) were significantly increased in jugular veins of the RHRSP group. Furthermore, endothelial p-Cav-1 and Eph-B4 were found to be diminished in jugular veins of the RHRSP group. Arterial hypertension caused venous collagenosis in the jugular veins. These changes may be strongly related to Eph-B4 and its downstream factor Cav-1/p-Cav-1.

Keywords: Hypertension venous remodeling, venous collagenosis, Eph-B4/caveolin-1 pathways

Introduction

Cerebrovascular disease remains the leading cause of death, worldwide, and hypertension is one of the primary causes of cerebrovascular disease. At the same time, vascular remodeling is the most primary pathological mechanism caused by hypertension. A previous study [1] observed changes in the cerebral veins of stroke-prone renovascular hypertensive rats (RHRSPs) using susceptibility-weighted imaging (SWI) and histopathological methods, finding that long-term hypertension in RHRSPs leads to increased visibility of cerebral veins on SWI and thickened cerebral venous walls (venous collagenosis). Thickened walls of the affected veins in RHRSPs may be ascribed to several conditions that result from hypertension. However, how arterial hypertension affects the venous system, effects on venous

remodeling, and mechanisms responsible for these changes remain unknown.

Eph-B4 is a member of the Eph receptor tyrosine kinase (RTK) family. It has been described as a marker of venous endothelial cell (EC) determination in embryonic development of diverse species, including mice, chicks, and so forth [2]. Kudo et al. [3] found that Eph-B4 was regulated during vein graft adaptation in humans and aged rats and that venous identity was preserved in the veins of aged animals, but lost during adaptation to arterial circulation. These results demonstrated that markers of vessel identity are plastic and that their selective regulation may mediate structural and functional changes of veins in aged animals and humans. Thus, whether “vein marker” Eph-B4 plays a role in jugular venous changes caused by arterial hypertension is questioned.

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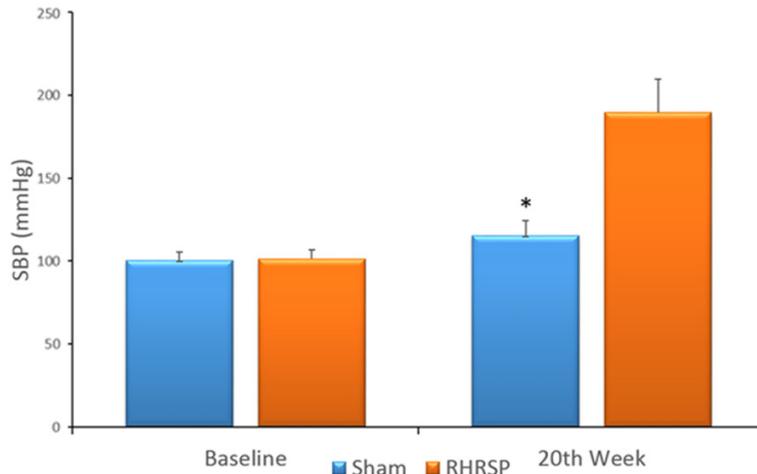


Figure 1. SBPs before and after surgery in the two groups (n=28). Baseline: before surgery; 20th week: 20 weeks after surgery. *P < 0.05 vs SBP of the RHRSP group in 20th week. Error bars denote SEM.

To resolve this issue, further research was performed on jugular veins using an RHRSP model, examining the effects and mechanisms of altered Eph-B4 signaling.

Materials and methods

Animal treatment

All animal procedures were approved by the Sun Yat-Sen University (Guangzhou, China) Committee for the Care and Use of Animals. A total of 80 male Sprague-Dawley rats, weighing 80-100 g, were obtained from the Experimental Animal Center of Guangdong Province and were randomly divided into the following 2 groups: sham-clipped group (sham, n=28) and RHRSP model group (RHRSP, n=52).

These male Sprague-Dawley rats were fed ad libitum and housed in conventional conditions with controlled temperature (23±2°C), humidity (55±10%), and light (12-hour light/12-hour darkness). All animal were treated in strict accordance with International Ethical Guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

An RHRSP rat model was used to induce renovascular hypertension [4-6]. Under anesthesia with 3% sodium pentobarbital (36 mg/kg body wt IP), a midline laparotomy was used for bilateral placement of partially occlusive silver clips (0.3 mm internal diameter) on the renal arteries of RHRSP rats. The ring part of the clip was placed around the root of each artery and the outer gap of the clip was then shut. Rats in the

sham surgery group underwent laparotomies and isolation of the bilateral renal arteries was without clip placement. After 4 weeks of recovery from surgery, systolic blood pressure (SBP) was measured with a tail cuff once per week.

Western blot analysis

The cortex of the parietal region of the brain was used for Western blots. This region matched that used for two-photon observations. Jugular veins were also used for Western blots. Samples containing equivalent amounts of ex-

tracted protein were loaded onto 10% gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were blotted using the following primary antibodies: caveolin-1 (R&D Systems, Minneapolis, USA), EphB4 (Santa Cruz Biotechnology, California, USA), and phosphorylated caveolin-1 (p-Cav-1, Santa Cruz Biotechnology, California, USA).

Real-time quantitative PCR

The cDNA sequence of rat EphB4 was purchased from Open Biosystems. The following primers were used for real-time PCR: GAPDH forward, 5'-GGCCTCCAAGGAGTAAGAAA-3' and GAPDH reverse, 5'-GCCCTCCTGTTATTATGG-3'; and EPHB4 forward, 5'-GCTCGGAACATCTTG-GTCAA-3' and EPHB4 reverse, 5'-CCCAGGG-AACTTGTGTAGGT-3'. PCR conditions were as follows: one cycle at 94°C for 4 minutes, followed by 30 cycles at 94°C for 30 seconds, and 60°C for 30 seconds. Amplification was quantified using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany). Quantified results for individual cDNAs were normalized to GAPDH using the $\Delta\Delta Ct$ method. Purities of the amplified products were examined using dissociation curves.

Immunohistochemistry and immunofluorescence

Samples were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5- μ m cross sections. Hematoxylin and eosin (H&E) and Masson trichrome staining were performed for all samples.

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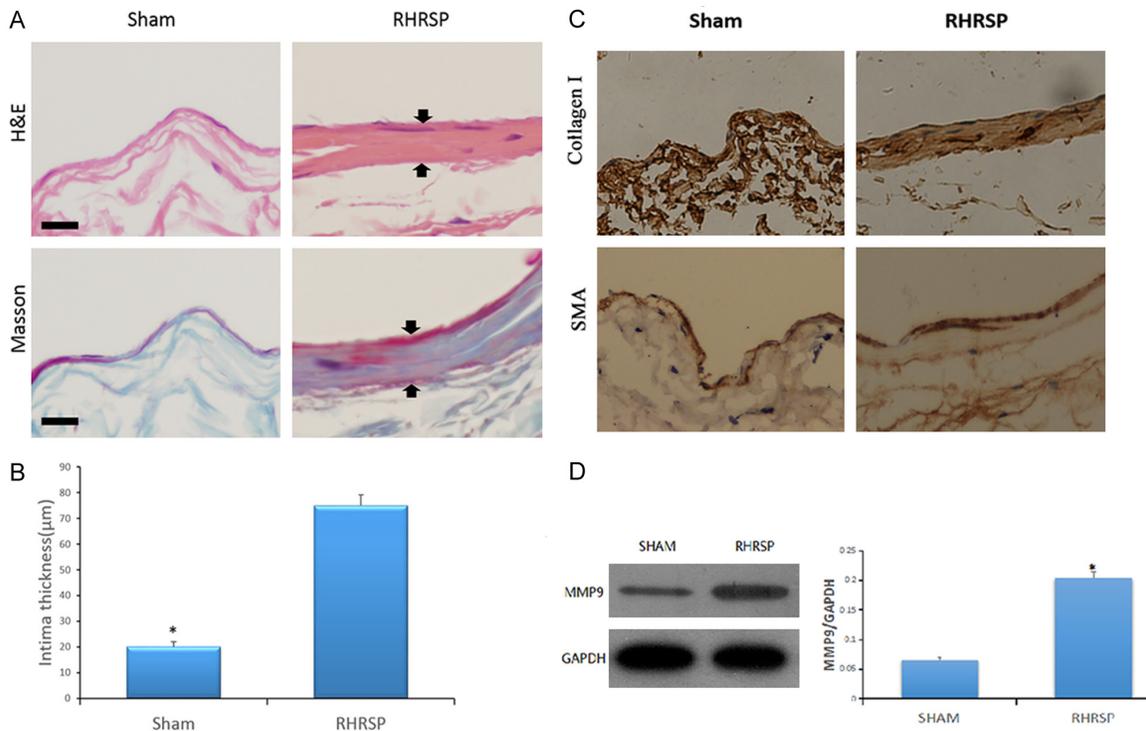


Figure 2. Jugular veins thickened in the RHRSP group and the walls were mainly composed of collagen I. MMP9 expression increased in the jugular veins (n=5). A. H&E staining demonstrating normal thin walls of the sham group and thickened walls of jugular veins in the RHRSP group. Masson trichrome staining illustrated that thickened walls in RHRSP group were composed of collagen. Arrows show intimal thickening. Bar, 100 μm. B. Jugular vein intima thickness of two groups. C. The type I collagen and smooth muscle actin (SMA) in the walls of jugular veins of both groups. D. MMP9 expression increased in jugular veins of the RHRSP group compared to the sham group. *P < 0.05. Error bars denote SEM.

Primary antibody treatments were performed, according to manufacturer instructions. Experiments for antibody validation and documented controls were carried out (**Figure 1**). Concentrations of all antibodies were optimized as needed. Primary antibodies included Cav-1, p-Cav-1, and Eph-B4. Antigen retrieval was performed using 10-mmol/L citrate buffer at pH 6.0. For immunohistochemistry, secondary detection was performed using DAB. Sections were counterstained with Mayer's hematoxylin. For immunofluorescence, secondary detection was performed using Alexa Fluor 488 and 568 and DAPI counterstain (Sigma, St. Louis, MO, USA). Images were captured with a fluorescent microscope under identical conditions.

Statistical analysis

SPSS 19.0 was used for statistical analyses. Data are expressed as mean ± SD. Student's t-tests were used to compare pairs of groups. P values < 0.05 are considered statistically significant.

Results

Of the 80 Sprague-Dawley rats, 4 rats died during the experiment in the RHRSP group. The sham group consisted of 28 normal healthy rats, used as a reference control group.

RHRSP model was established successfully

SBP increased gradually in all RHRSP rats but did not significantly increase in the sham group. Compared to baseline and sham group values, SBPs of the RHRSP group were significantly increased 4 weeks after surgery. Twelve weeks after the RHRSP rats underwent surgery, increased SBPs were maintained at a relatively higher and stable level (**Figure 1**, p < 0.05).

Jugular veins thickened in the RHRSP group and walls were mainly composed of collagen I

H&E staining demonstrated normal thin walls of jugular veins in the sham group and thickened walls of jugular veins in the RHRSP group

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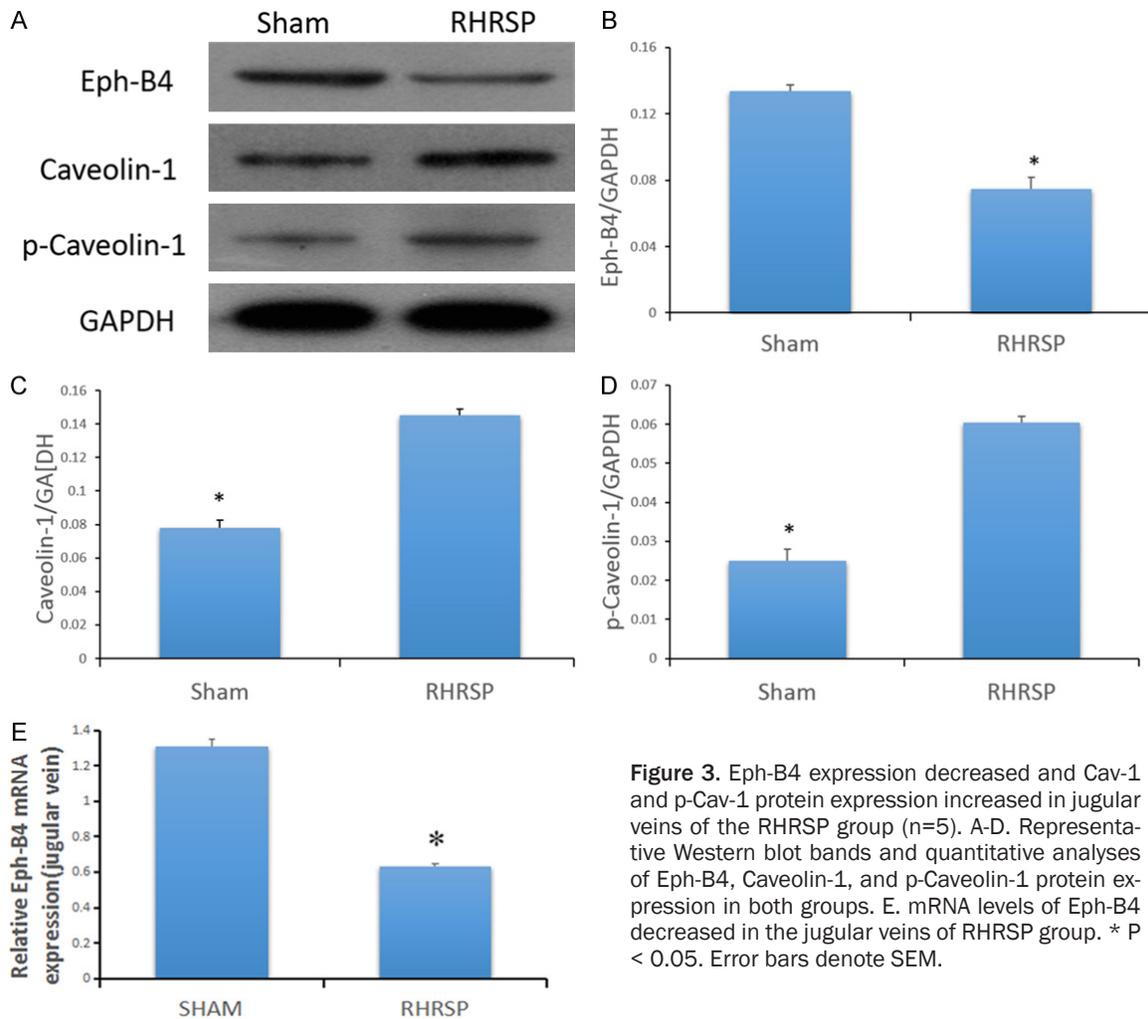


Figure 3. Eph-B4 expression decreased and Cav-1 and p-Cav-1 protein expression increased in jugular veins of the RHRSP group (n=5). A-D. Representative Western blot bands and quantitative analyses of Eph-B4, Caveolin-1, and p-Caveolin-1 protein expression in both groups. E. mRNA levels of Eph-B4 decreased in the jugular veins of RHRSP group. * P < 0.05. Error bars denote SEM.

(**Figure 2A**). Masson trichrome staining revealed that these thickened walls were composed of collagen (**Figure 2A**). Intimal thickness of jugular veins was measured for both groups. Differences between the two groups were significant (**Figure 2B**).

To explore the main constituent of the thickened walls of jugular veins, this study dealt with jugular veins of both groups using immunohistochemistry staining (**Figure 2C**). It was found that the thickened walls mainly consisted of type I collagen, however, no significant differences were detected concerning expression of smooth muscle actin (SMA) in the jugular veins between the sham group and RHRSP group.

MMP9 expression increased in jugular veins of the RHRSP group compared to that of the sham group

Protein expression of MMP9 was examined in jugular veins via Western blotting, finding that it

was significantly increased in the RHRSP group, compared to the sham group (**Figure 2D**).

Eph-B4 expression decreased and Cav-1 and p-Cav-1 expression increased in jugular veins of the RHRSP group

Levels of Eph-B4, Cav-1, and p-Cav-1 proteins were detected by Western blot in the jugular veins of sham and RHRSP groups. Compared to the sham group, protein expression of Eph-B4 was significantly decreased and levels of Cav-1 and p-Cav-1 were significantly increased in jugular veins of the RHRSP group (**Figure 3A-D**).

Next, mRNA levels of Eph-B4 were examined in the jugular veins of both groups using quantitative real-time PCR. The RHRSP group exhibited less detectable Eph-B4 than the sham group, suggesting that expression of Eph-B4 transcripts was downregulated in the hypertensive rats (**Figure 3E**).

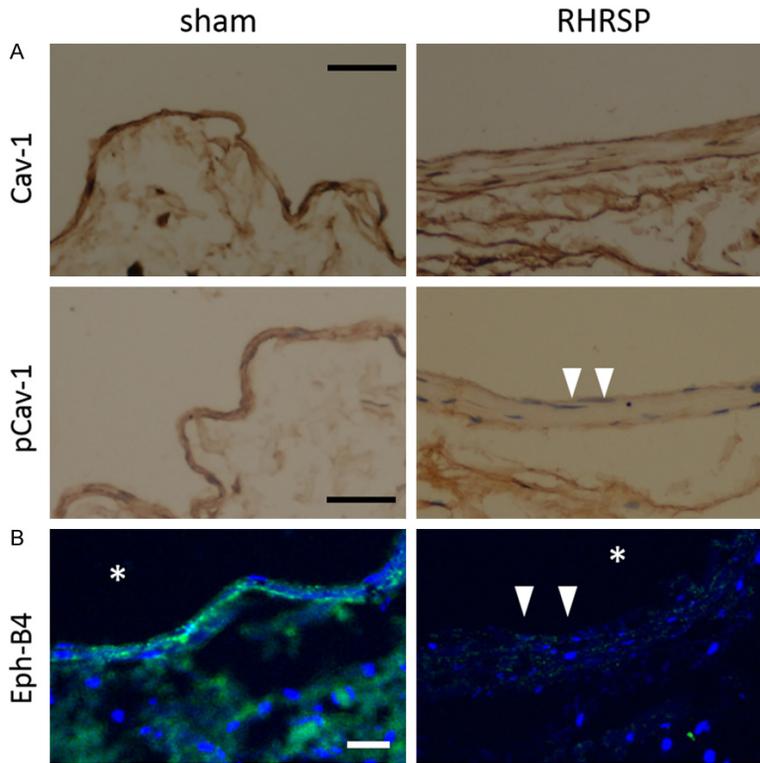


Figure 4. Endothelial p-Cav-1 and Eph-B4 was diminished in jugular veins of RHRSP group (n=5). A. Immunohistochemistry against Cav-1 and p-Cav-1, Bar, 200 μ m. Arrowheads denote loss of p-Cav-1 signal; B. Immunofluorescence against Eph-B4, Bar, 100 μ m. Arrowheads denote loss of Eph-B4 signal. *, lumen.

Eph-B4 and p-Cav-1 expression diminished in endothelial cells of jugular veins of the RHRSPs

Immunohistochemistry demonstrated that endothelial Cav-1 was detectable in jugular veins of both groups, but endothelial p-Cav-1 was strongly diminished in the RHRSP group (**Figure 4A**). Immunofluorescence showed that Eph-B4 was diminished in jugular veins of the RHRSP group (**Figure 4B**).

Discussion

In a previous study [1], it was proven that arterial hypertension affects the venous system of the brain. Increased visibility of cerebral veins on SWI and thickened cerebral venous walls (venous collagenosis) were both consequences of long-term hypertension in RHRSPs. Furthermore, it was validated that stenosis in these affected veins led to slower velocities, resulting in disordered venous drainage. The present study investigated venous remodeling in jugular veins of the RHRSP group, examining

possible mechanisms. It was found these changes may be strongly related to Eph-B4 and its downstream factor Cav-1/p-Cav-1.

A previous study by Bruno reported that an aged group displayed an increment of collagen fibers versus a young group. Moreover, even in the youngest group, hypertension accelerated collagen deposition in the small arteries. Histopathology results in the present study indicated that hypertension also caused venous collagenosis, rather than smooth muscle cell mitogen secretion, in jugular veins of the RHRSPs. This was in accord with venous collagenosis observed in a previous study. Bruno's study found that collagenosis may cause jugular veins to become less distensible and slower to relax, impairing blood flow from the brain back to the heart. This process leads to increased circumferential wall

tension (CWT), an important determinant of vascular remodeling. Moreover, it was found that expression of MMP9 was increased in the jugular veins of hypertensive rats. Thus, it was hypothesized that elevated blood pressure leads to overload capacity of the body, increasing CWT. This, on one hand, stimulates the proliferation of vascular smooth muscle cells and collagen. On the other hand, it promotes MMP9 secretion which reduces the elasticity of blood vessels. The pathologic changes above, in turn, increase CWT, forming a vicious circle and leading to venous remodeling.

Remodeling of jugular veins in hypertensive rats (i.e., venous collagenosis) is substantially different from the corresponding process in arteries. This elicits the question, through which signaling pathways does venous remodeling occur?

During embryonic development, Eph receptors and their membrane-bound ephrin ligands play essential roles in the formation of functional vascular networks [7-9]. Eph-B4 is an

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active determinant of embryonic venous development [2, 10-12]. It is also present in adult veins where it provides a marker of venous identity. Kudo [3] used immunofluorescence to confirm that Eph-B4 is expressed in aged rat jugular veins in both the endothelium and α -actin-positive medial SMCs. He also confirmed that Eph-B4 is strongly diminished, but not eliminated in the intima media of venous grafts placed in the relatively hypertensive environment of arterial circulation. The present study obtained similar results. Eph-B4 signaling was reduced in jugular veins of the hypertensive rats, as was signaling in the intima. Thus, it was questioned whether reduced Eph-B4 expression is responsible for venous remodeling in hypertensive rats.

A study in 2011 [13] demonstrated that the loss of Eph-B4 during venous transposition to the arterial circulation was directly responsible for the loss of normal venous structures and excessive venous wall thickening and remodeling. Lynn S. Model et al. [14] examined changes in vessel identities of human saphenous veins in a flow circuit in which shear stress could be precisely controlled. They found that venous Eph-B4 expression was diminished and expression of osteopontin was increased with exposure to arterial magnitudes of shear stress. These authors concluded that arterial magnitudes of shear stress cause the loss of venous identity. To increase knowledge of the function of Eph-B4, a study in 2013 found that diminished venous endothelial Eph-B4 expression was associated with an angiogenic and mitogenic phenotype that is characterized by increased secretion of smooth muscle cell mitogens and reduced nitric oxide production [15]. This result suggests that decreased Eph-B4 expression is related to vascular remodeling.

Cav-1 is a major structural protein of the caveolae in endothelial cells. It is thought to be involved in the mechanotransduction of dynamic shear stress changes via interactions with several signaling protein families, including Eph receptors [16, 17]. Forrester's research [18] found that Cav1(-/-) mice, infused with AngII, showed attenuation of medial thickness and perivascular fibrosis in the thoracic aorta, indicating that dysfunction of Cav-1 leads to vascular remodeling. Moreover, a study by Muto [13] suggested that Cav-1 is downstream of

Eph-B4 signaling during venous adaptation. This author examined vein graft adaptation in WT and Cav-1 KO mice, finding that vein grafts derived from Cav-1 KO mice exhibit significantly increased wall thickness, like that observed in vein grafts with reduced Eph-B4 function. However, vein grafts derived from Cav-1 RC mice, such as Cav-1 KO mice with an EC-specific Cav-1 transgenic reconstitution, exhibited greatly reduced thickness compared with vein grafts derived from Cav-1 KO mice. These results suggest that the thickening response is a result of endothelial Cav-1, not global Cav-1, dysfunction and that endothelial Cav-1 is essential to the Eph-B4 signaling that limits venous wall thickness. The present study further observed increased Cav-1 and p-Cav-1 in the thickened jugular veins of RHRSPs at both protein and mRNA levels. This seemed to contradict the findings that Eph-B4 stimulated phosphorylation of Cav-1 in the endothelial cells [13] and that Eph-B4 was greatly reduced in RHRSPs. Furthermore, immunohistochemistry was used to detect endothelial Cav-1 and p-Cav-1. It was found that endothelial Cav-1 was detectable in both groups, but endothelial p-Cav-1 was strongly diminished in the RHRSP group, in accord with Mito's results. Results also suggest that probably only p-Cav-1 in the endothelial cells is functional active and plays a role in vascular remodeling. Decreased p-Cav-1 in the endothelium may have been caused by the reduction in Eph-B4 in the RHRSP group.

In conclusion, the present study found that arterial hypertension caused venous collagenosis but no change of expression of smooth muscle actin in jugular veins. Moreover, these changes may be strongly related to Eph-B4 and its downstream factor Cav-1/p-Cav-1, especially the active protein p-Cav-1 in endothelial cells. Until now, substantial research in this field has not focused on target protein expression of special delicate structures, including endothelial cells and the smooth muscle layer. Studies have reported that Cav-1 can inhibit vascular remodeling through reducing superoxide production and vascular smooth muscle cell proliferation [19]. However, specific mechanisms of venous remodeling in the environment of arterial hypertension are still ambiguous. Thus, further research is required to explore this big problem.

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Disclosure of conflict of interest

None.

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