EP3 Receptor Deficiency Attenuates Vascular Remodeling and Cognitive Impairment in a Cerebral Small Vessel Disease Model

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Research

Keywords: cerebral small vessel disease, hypertension, E prostanoid receptor 3, transforming growth factor beta1, extracellular matrix

Posted Date: October 26th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-95403/v1>

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Abstract

Background

Anti-hypertensive therapy has achieved effective but not complete results in treating cerebral small vessel disease (CSVD), suggesting the necessity for additional treatments. Targeting abnormal inflammatory responses has become a research interest. Inhibition of E-prostanoid 3 (EP3) receptor has been shown to attenuate vascular remodeling in peripheral organs, but little is known about its role in CSVD. Here, we investigated whether the deletion of *EP3* attenuates the development of CSVD.

Methods

Stroke-prone renovascular hypertensive rat (RHRsp) was used as an animal model of CSVD. The effect of *EP3* deletion on the expression of extracellular matrix (ECM) in cerebral small arteries of RHRsp was detected by immunofluorescence. Changes in cognitive function and cerebral blood flow (CBF) of RHRsp were evaluated using the Morris water maze test and MRI. In vitro experiments with primary rat brain microvascular smooth muscle cells were used to confirm whether the transforming growth factor beta 1 (TGF- β 1) signal takes part in the process.

Results

Cerebral small arteries of RHRsp exhibited increased EP3 expression. Despite no alleviation in hypertension, the deletion of *EP3* still reduced the overexpression of ECM in the cerebral small arteries of RHRsp. In vitro experiments indicated that *EP3* deletion regulated the expression of ECM by downregulating TGF- β 1/Smad signaling. *EP3* knockout further attenuated the cognitive impairment of the RHRsp possibly through increased CBF.

Conclusion

Together, our results indicate that the deletion of *EP3* attenuates the vascular remodeling and vascular cognitive impairment induced by hypertension, and blockade of the EP3 receptor may be a promising strategy for the treatment of CSVD.

Background

Cerebral small artery remodeling is a main pathological feature of arteriolosclerotic cerebral small vessel disease (CSVD), characterized by lumen narrowing and abnormal accumulation of extracellular matrix (ECM) in the vessel wall [1]. Cerebral small artery remodeling reduces cerebral blood flow (CBF) leading to hypoperfusion of the brain [2, 3], which is closely related to the subsequent development of cognitive impairment [4, 5]. Hypertension is a major risk factor for CSVD [6], and chronic hypertension triggers vascular remodeling in CSVD. However, recent clinical studies also reveal that despite anti-hypertensive medication, some patients still present progressive vessel pathology and cognitive disorder [7, 8]. Given

this, additional treatment apart from anti-hypertension targeting vascular remodeling in CSVD is becoming increasingly important.

Inflammation has long been listed as a candidate factor in the development of CSVD[9], and tackling abnormal inflammatory responses has become a potential treatment target for CSVD. One of the research focuses is cyclooxygenase 2 (COX-2), a key enzyme in prostaglandin biosynthesis. A study showed that a *COX-2* genetic polymorphism in the Chinese population may contribute to the risk of developing white matter lesions[10]. Our previous study also revealed that using COX-2 inhibitor can attenuate CSVD in an animal model[11]. However, chronic use of COX-2 inhibitors also results in adverse side effects and increases the risk of vascular diseases possibly by inhibiting the endothelial prostacyclin production[12], leading us to consider that modulating the prostaglandins and prostaglandin receptors downstream of the COX-2 pathway would be a much better strategy.

Among the prostaglandins synthesized from COX-2, prostaglandin E₂ (PGE₂) is the main product produced during inflammatory processes. PGE₂ has four receptor subtypes, namely, E prostanoid (EP) receptors (EP1–4) [13]. Elevated expression of EP3 receptors has been reported in the small arteries of hypertensive animals[14], and inhibition of EP3 receptors has been shown to reverse cardiac hypertrophy induced by hypertension[15] or attenuated pulmonary arteriole remodeling in pulmonary hypertension[16]. However, the role of EP3 receptors in the cerebral small artery remodeling of CSVD induced by hypertension is still unknown.

With this background, this study aimed to evaluate the role of EP3 receptors in CSVD, using a renal hypertensive animal model– stroke-prone renovascular hypertensive rat (RHRsp). The RHRsp model is established by a two-kidney, two-clip(2k2c) procedure[17]. After the operation, RHRsp presents with persistent hypertension. Studies have shown that RHRsp rats presented cerebral small artery remodeling with fibrinoid necrosis, hyalinosis, and apparent luminal narrowing[18]. Behavioral tests also confirmed cognitive impairment in RHRsp[19]. These results suggest that RHRsp is a relatively ideal animal model of CSVD, especially for the study of vascular pathological changes in hypertension-associated CSVD.

Methods

Animals and Treatment

Because of the possible effect of sex hormones on hypertension induced by two-kidney, two-clip (2k2c) procedure, experiments were performed in male rats. Animals were housed in rooms under conditions of controlled temperature (21±2°C), humidity (60±10%), and a 12 h light/dark cycle. *EP3*^{-/-} (*EP3* knockout) rats in a Sprague-Dawley (SD) background were created by Beijing Biocytogen (Beijing, China) using CRISPR/Cas9-based technology (detailed description provided in the Expanded Materials and Methods). Wild-type (WT) littermates (*EP3*^{+/+} rats) were generated as experimental controls from *EP3* receptor heterozygous mating. We used 30 *EP3*^{-/-} rats and 30 WT rats in this study. Rats were subjected to either a two-kidney, two-clip (2k2c) procedure to establish the RHRsp model or a sham surgery for control group.

The operation details are provided in the Supplementary Material. Investigators responsible for functional assessment, outcome measurement, and data analysis were blinded to experimental groups.

Blood Pressure (BP) monitoring

BP measurements were performed in adult *EP3^{-/-}* and WT rats aged 12 weeks. Two months and four months after the 2k2c procedure or sham operation, the BP of the animals was also measured.

Conscious BP measurement using the tail-cuff method (BP-98A; Softron Co, Tokyo, Japan): the day before blood pressure was measured, animals were induced into the holding cage for 15 min in order to help the animals adapt to the environment. On the day of measurement, the instrument was preheated for 15 min before use, and then the rats were induced into the holding cage, with its tail being properly put into the pressurized sensor sleeve. Blood pressure was measured only after the animals calm down in the cage. The measurement was repeated three times at intervals greater than five min, and the average of each animal was taken. **Unconscious BP measurement** using invasive carotid artery catheterization: the rats were anesthetized with sodium pentobarbital and placed on a temperature-controlled pad. One side of the carotid artery was exposed and a polyethylene tube prefilled with heparinized normal saline was inserted into the artery, and BP was measured by a pressure transducer and recorded in real-time.

Evaluation of CBF and Cognitive Function

Dynamic susceptibility contrast perfusion-weighted imaging (DSC-PWI) was conducted to evaluate the CBF of animals. The Morris water maze test was conducted 16 weeks after the 2k2c operation to evaluate the cognitive function of the animals. The detailed methods are provided in the Supplementary Material.

Brain Tissue Preparation

The rats were sacrificed 4-5 months after surgery and transcardially perfused with approximately 300 mL of 0.9% saline, followed by 200 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS, pH 7.4) within 5 min. Then, the brain tissues were post-fixed in 4%PFA for 4-6h and stored in 30% sucrose in 0.1 M PBS (pH 7.4) until the brain tissues fell to the bottom of the solution. The brains were sectioned into coronal serial slices (10µm thickness) from about +5 mm bregma to -5 mm bregma.

Immunofluorescence

Primary antibodies against EP3(Sigma), smooth muscle actin alpha (αSMA, Sigma), collagen I (Abcam), collagen IV (Abcam), fibronectin (Abcam) and laminin (Abcam) were used. The slides were photographed using a fluorescence microscope (Olympus, BX-51, Tokyo, Japan). For each primary antibody, three sections per animal were stained. We divided each cross-section of the brain into three parts according to a previous study[20]: cortical gray matter, white matter, and deep gray matter. At least three random fields of each part were selected and photographed for further analysis. The cerebral small arteries (10-65µm)

were indicated by the immunostaining of α SMA[19]. The detailed methods are provided in the Supplementary Material.

Cell culture

Primary rat brain microvascular smooth muscle cells (BMVSMCs) were cultured from male 5-day-old rats by modification of the procedure described previously[21, 22] (Detailed description provided in the Supplementary Material). Immunocytochemistry with anti- α SMA was used for the identification of vascular smooth muscle cells(VSMCs). Before stimulation, rat BMVSMCs were growth-arrested by incubation in serum-free medium for 24 h. Angiotensin II (ANGII, Sigma), LY364947 (Selleck), and L798106 (Cayman) were used for the stimulation.

Western blotting

The concentration of proteins extracted from rat BMVSMCs was determined using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). Equal quantities of proteins were denatured and resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, incubated with 5% skimmed milk for 1 h, and then incubated with primary antibodies overnight at 4°C. The membranes were then conjugated with a horseradish peroxidase-labeled secondary antibody for 1 h at RT. Blots were developed using an enhanced chemiluminescence reagent (Thermo Scientific), followed by densitometric quantification using ImageJ.

Statistical Analysis

Prism 8.0 software was used for data analysis. Data are presented as the mean $\pm$ standard deviation. All data sets were normally distributed, and variance was homogeneous among all groups as determined by one-way analysis of variance. Differences between two groups were analyzed using a t-test and data among multiple groups were compared using one-way analysis of variance. Statistical significance was set at $p < 0.05$.

Results

EP3 is upregulated in cerebral small arteries of RHRsp, and deletion of *EP3* does not affect blood pressure

COX-2/ PGE2 signaling is an important inflammatory pathway. Hypertension is strongly associated with the augmented expression of the COX-2/ PGE₂/EP3 axis in both human and animal models[14, 15]. As such, we also observed significantly elevated expression of EP3 in the cerebral small arteries of RHRsp by immunofluorescence (Figure 1A), indicating that the PGE2/EP3 axis might be involved in the progression of CSVD induced by hypertension.

We then constructed the RHRsp model on *EP3* knockout rats (*EP3*^{-/-}) to determine whether EP3 receptor deficiency can attenuate the development of CSVD. First, we sought to determine whether the deletion of

EP3 can affect the BP of the animals since hypertension is a crucial risk factor for CSVD and the PGE₂ signaling is reported to have major effects on BP control through its receptors[23]. We used two methods to monitor BP, including conscious BP measurement using the tail-cuff method and unconscious BP measurement using the invasive carotid artery catheterization. Results showed no significant difference in the baseline BP of either group (Figure 1B). Furthermore, we measured the BP of the rats after the 2k2c procedure. Both *EP3*^{-/-} rats and the WT rats showed significantly increased BP compared to the sham-operated groups. However, there was still no difference between *EP3*^{-/-} rats and the WT rats within the hypertensive group (Figure 1B), indicating that *EP3* knockout did not impact both baseline BP and the induction of hypertension by the 2k2c procedure.

Deletion of *EP3* attenuates the overexpression of ECM in the cerebral small arteries of RHRsp

Next, we tested whether disruption of *EP3* modulates the progression of cerebral small artery remodeling in RHRsp. Two months after the 2k2c procedure, rats in the RHRsp group developed stable hypertension. By the end of 4-5months, the cerebral small arteries of RHRsp showed a significantly higher expression of ECM including fibronectin, laminin, collagen I, and collagen IV, compared with that observed in controls with normal BP. Intriguingly, *EP3*^{-/-} RHRsp rats displayed a significant reduction in all four kinds of ECM, compared with that in WT RHRsp (Figure 2). These results indicated that despite no effect on hypertension, the deletion of *EP3* still attenuated the overexpression of ECM in the cerebral small arteries of RHRsp.

Deletion of *EP3* decreases the expression of ECM in BMVSMCs under AngII stimulation

As shown in above, our results confirmed that the deletion of *EP3* successfully attenuated cerebral artery remodeling in RHRsp without alleviating high blood pressure. Hence, we assumed that *EP3* may regulate the expression of ECM directly by affecting VSMCs. To further study the mechanism by which *EP3* regulate VSMCs to express ECM in CSVD, BMVSMCs were extracted from both WT rats and *EP3*^{-/-} rats. To mimic the elevated expression of ECM in vitro, we used ANGII to stimulate BMVSMCs, since both hypertensive humans and animals including RHRsp were reported to have an elevated level of ANGII[24, 25]. As shown in Figure 3, under the stimulation of ANGII, BMVSMCs exhibited significantly higher ECM expression including fibronectin, laminin, collagen I, and collagen IV. We found that the addition of the *EP3* inhibitor L798106 could significantly alleviate this increased ECM expression induced by ANGII. Furthermore, the deletion of *EP3* genes can significantly inhibit the baseline expression of ECM (without ANGII stimulation), and even under the stimulation of ANGII, a significantly higher expression of ECM was not observed in *EP3*^{-/-} rat BMVSMCs. These results suggest that *EP3* gene knockout can reverse the increased expression of ECM induced by ANGII in rat BMVSMCs.

Deletion of *EP3* decreases the expression of ECM by suppressing TGF-β1 signaling in BMVSMCs under ANGII stimulation

TGF- β 1 signaling has long been reported to play a role in regulating ECM expression[26]. It has also been suggested that knockout of *EP3* downregulates the expression of ECM in pulmonary arterioles by suppressing the TGF- β 1/Smad pathway[16]. In this study, we sought to determine whether EP3 can regulate the expression of ECM by altering the TGF- β 1 signaling. As shown in Figure 4A-B, western blot analyses confirmed that the level of TGF- β 1 and phosphorylation of Smad2/3 were elevated in rat BMVSMCs under ANGII stimulation. Inhibiting TGF- β 1 signaling using LY364947 attenuated the high expression of ECM induced by ANGII in rat BMVSMCs (Figure 4A). Furthermore, the deletion of *EP3* rescued ANGII-induced activation of TGF- β 1 signaling, as evidenced by a decreased level of TGF- β 1 and phosphorylation of Smad2/3(Figure 4B). These results indicated that the deletion of *EP3* can decrease the expression of ECM by suppressing TGF- β 1 signaling in BMVSMCs under ANGII stimulation.

Previous studies have shown that the EP3 receptor modulates multiple intracellular signaling pathways by coupling different types of heterotrimeric G proteins[27]. As shown in Figure 4C, the downregulation of ECM by EP3 inhibition was abolished by pretreatment with pertussis toxin (PTX) in BMVSMCs, indicating that EP3 signaling regulated the expression of ECM via the PTX-sensitive G protein, Gi/o in BMVSMCs.

Deletion of *EP3* attenuates decreased CBF in RHRsp

Cerebral small artery remodeling is the leading cause of decreased CBF in patients[28]. In order to determine whether attenuated vascular remodeling caused by *EP3* deletion can further improve CBF in RHRsp, we used PWI to monitor the global CBF of the animals. T2-weighted images and representative CBF images of the animals are shown in Figure 5. Consistent with previous studies on *EP3*^{-/-} mice[29] or mice treated with selective antagonists of EP3[30], our results showed no difference in CBF between normal WT rats and *EP3*^{-/-} rats. As decreased CBF is seen in CSVD patients[31], our results showed that RHRsp demonstrated a lower global CBF, compared to the control rats. Intriguingly, *EP3*^{-/-} RHRsp rats displayed a recovery of global CBF, compared with that in WT RHRsp. These results indicate that the deletion of *EP3* can improve CBF in RHRsp, confirming our hypothesis that the deletion of *EP3* can not only reverse vascular structure changes but also contribute to the functional recovery of blood supply in the brain.

Deletion of *EP3* attenuates cognitive impairment in RHRsp

As seen above, the deletion of *EP3* successfully attenuated vascular remodeling in RHRsp and improved the cerebral blood supply of the animals. We assume that these improvements may also help to attenuate the cognitive impairment in RHRsp. To this end, we used the Morris water maze test to evaluate the cognitive function of the animals. The Morris water maze test is designed to reflect the spatial learning and memory ability. During the hidden platform acquisition phase, on average, rats in all groups showed a progressive decrease in escape latency, indicating that subjects could learn the location of the platform. However, compared with the sham-operated group, RHRsp rats had a longer escape latency, and a statistical difference appeared on the third and fifth days. The deletion of *EP3* (*EP3*^{-/-}RHRsp) shortened the escape latency. However, there was no statistically significant difference (Figure 6). A no-platform

probe test was conducted 24 h after the final day5 training trial. Figure 6 shows that the time spent in the target quadrant was decreased in RHRsp rats, compared with the sham-operated group. The deletion of *EP3* (*EP3*^{-/-} RHRsp) reversed this effect. The average number of times crossing the platform was higher in RHRsp than in sham-operated rats, and the deletion of *EP3* (*EP3*^{-/-} RHRsp) significantly improved the number. Taken together, these results show damaged spatial learning and memory ability in RHRsp, and the deletion of *EP3* in RHRsp can rescue cognitive impairment. We cautiously speculate that the improvement of cognitive function in *EP3*^{-/-}RHRsp could be partially attributed to the reduced vascular remodeling and increased CBF by the deletion of *EP3*.

Discussion

In this study, we first observed increased EP3 expression in the cerebral small arteries of an animal model of CSVD. Then, we demonstrated that *EP3* gene knockout can reverse cerebral small artery remodeling, possibly by downregulating TGF- β 1 signaling. Furthermore, we found that vascular structure improvement by *EP3* deletion also attenuates the decreased CBF of RHRsp and eventually yield success in reducing the cognitive impairment of the animals.

Our results revealed the possibility to improve vascular remodeling in CSVD through *EP3* deletion, while independent of controlling hypertension. Increased COX-2/PGE₂/EP3 signaling has long been observed in hypertensive patients and animals, and inhibition of the signaling pathway attenuates the vascular remodeling in hypertension [14, 15]. Although COX-2/PGE₂/EP3 signaling is found to affect BP[23], the conclusion is conflicting on whether the improvement of vascular remodeling by inhibitions of COX-2/PGE₂/EP3 signaling in vivo is accompanied by a decrease in hypertension[11, 15, 32]. The possible reasons are that different hypertension induction methods are used in these studies and the genetic backgrounds of the animals are distinct[32]. In this study, our results indicated that *EP3* knockout did not affect the baseline BP of the rats, and previous studies on *EP3*^{-/-} mice suggest the same outcome[29, 33]. Although the *EP3* gene knockout was reported to relieve hypertension caused by ANGII perfusion in mice[34] or salt-induced hypertension in mice[33], our results suggest that *EP3* gene knockout did not affect the hypertensive response induced by the 2k2c operation. These results may indicate that the mechanism of persistent hypertension in RHRsp is much more complex. After the surgery, as juvenile rats grow, the renal arteries were gradually narrowed by the fixed-size silver clamps, gradually making the rats' bilateral kidneys ischemic. Continuous renal ischemia activates the renin-angiotensin system and increases the release of sympathetic neurotransmitters, endothelin, aldosterone, and other substances, thereby maintaining the persistence of hypertension[17, 25]. Our results further confirm the existence of hypertension independent mechanisms behind vascular remodeling in hypertension-induced CSVD, which are promising targets for CSVD treatments.

Since the deletion of *EP3* attenuated the ECM over-expression in the cerebral small arteries of *EP3*^{-/-} RHRsp without relieving hypertension, we assumed that direct regulation of VSMCs by the EP3 gene could be crucial in the process. Our in vitro study later confirmed that *EP3* can directly regulate the

expression of ECM in VSMCs under the stimulation of AngII. A similar effect of EP3 on VSMCs has already been reported in pulmonary arteriolar smooth muscle cells[16], and research has also suggested that EP3 gene knockout can reduce the migration and polarity of VSMCs[35]. TGF- β 1 signaling has long been found to regulate ECM production. Studies have indicated an elevated level of TGF- β 1 in both hypertensive patient[36] and hypertensive animal models[37]. Our results demonstrated that the level of TGF- β 1 and phosphorylation of Smad2/3 were elevated in rat BMVSMCs under ANGII stimulation and were relevant to the high expression of ECM. The deletion of *EP3* can reverse increased TGF- β 1/Smad signaling and attenuate the high expression of ECM. Previous studies also revealed that different receptors of PGE₂(EP1-4) participate in the regulation of ECM expression by affecting TGF- β 1/Smad signaling. For example, studies have shown that the TGF- β 1 pathway is involved in regulating the expression of renal vascular ECM, which is closely related to the occurrence of renal fibrosis. Activation of EP1 and EP3 receptors promotes renal fibrosis and accelerates renal damage[38], while activation of EP2[39] and EP4[40] receptors can inhibit TGF- β 1-mediated increased expression of ECM in renal mesangial cells and alleviate renal damage. PGE₂ can also inhibit TGF- β 1 /Smad signaling via the EP2 receptor, thereby reducing the expression of various types of collagen as well as the formation of hypertrophic scars[41]. These results suggest that the role of the COX2/PGE₂/EP axis in vascular remodeling in diseases is a complex issue. In different tissues and organs or under different pathological conditions, the COX2/PGE₂ axis may regulate ECM expression through TGF- β 1 signaling by binding to different receptors.

In our study, the deletion of *EP3* further improved the CBF and cognitive function in RHRsp. We assume that this could be partially attributed to reduced vascular remodeling under EP3 deletion. However, our research cannot rule out other possible mechanisms in the process. PGE₂ acts as a vasodilator and vasodepressor through four distinct EP receptors, and stimulation of EP3 triggers processes leading to vasoconstriction[42]. It has been reported that activation of EP3 triggers vasoconstriction in porcine middle cerebral arteries[43]and in the guinea pig aorta[44]. It is possible that *EP3* deletion contributed to the improvement of CBF in RHRsp by reversing the enhanced vascular constriction under hypertension. Our study also witnessed an improved cognitive function accompanied by an increased CBF in *EP3*^{-/-} RHRsp. In addition to vascular protection, *EP3* deletion may preserve cognitive function in RHRsp through direct protection of neurons. Previous studies have indicated that blocking the EP3 receptor with L798106 rescued the expression of synaptic plasticity-related proteins in an animal model of surgery-induced memory deficits[45].

Conclusions

In conclusion, our study revealed that the deletion of the *EP3* gene can significantly improve the over-expression of ECM in cerebral small arteries of CSVD rats, leading to improved CBF and cognitive function, and the effect may partially through downregulated the TGF- β 1 /Smad signaling. Thus, blockade of the EP3 receptor may be a promising strategy for the treatment of CSVD.

Abbreviations

CSVD	cerebral small vessel disease
ECM	extracellular matrix
CBF	cerebral blood flow
COX-2	cyclooxygenase 2
PGE ₂	prostaglandins E 2
EP3	E prostanoid 3
RHRsp	stroke-prone renovascular hypertensive rat
αSMA	smooth muscle actin alpha
BMVSMCs	brain microvascular smooth muscle cells
TGF-β1	transforming growth factor beta1
ANGII	angiotensin II
BP	blood pressure

Declarations

Ethics approval and consent to participate

All animal use procedures were strictly performed in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Fudan University Experimental Animal Science Department Animal Welfare and Ethics Review Board.

Consent for publication

Not applicable.

Availability of data and materials

Please contact the author for data requests.

Competing interests

The authors declare that they have no competing interests.

Funding

Funding support was provided by the National Natural Science Foundation of China (81671151, Prof Fu), the National Natural Science Foundation of China (81901179, Dr Tang), the National Key Research and

Development Programs (2016YFC1300600, Profs Fu and Mok); the National Key Research and Development Programs(2016YFC1301603, Prof Fu).

Authors' contributions

FJH, WYL and MV conceived the study and designed experiments. LN and TJ performed the experiments and wrote the manuscript. LN, TJ, XY, ZMY and RX analyzed the results. All authors read and approved the final version of the manuscript.

Acknowledgements

Not applicable.

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Figures

Figure 1

(A) Immunofluorescence for EP3 (Green) and αSMA (Red) of SD and RHRsp. The cerebral small arteries (10-65μm) are indicated by the immunostaining of αSMA. Bar, 100μm. (B) Blood pressure in SD and RHRsp. SD, Sprague Dawley rat; RHRsp, Stroke-prone renovascular hypertensive rat; αSMA, smooth muscle actin alpha; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; m, month; **, $p \leq 0.01$; ****, $p \leq 0.0001$.

Figure 2

The deposition of extracellular matrix in the vessel wall of cerebral small arteries. (A) Immunofluorescence for αSMA (Green) and collagen IV (Red) of the animals. (B) Immunofluorescence for αSMA (Green) and laminin (Red) of the animals; (C) Immunofluorescence for αSMA (Green) and collagen I (Red) of the animals ; (D) Immunofluorescence for αSMA (Green) and fibronectin (Red) of the animals. The cerebral small arteries (10-65μm) are indicated by the immunostaining of αSMA. Bar, 100μm. RHRsp, Stroke-prone

renovascular hypertensive rat; αSMA, smooth muscle actin alpha, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 .

Figure 3

Change of extracellular matrix expression in rat BMVSMCs under different stimulation. (A)Representative western blots and quantification of the expression of fibronectin, laminin, collagen I and collagen IV in rat BMVSMCs under different stimulation. (B) Quantification of western blots of fibronectin, laminin, collagen I and collagen IV. BMVSMCs, brain microvascular smooth muscle cells; ANGII, Angiotensin II. *, p ≤0.05; **, p ≤0.01; ****, p ≤0.0001.

Figure 4

Quantification of western analysis. (A) TGF-β1 were elevated in rat BMVSMCs under ANGII stimulation. Suppressing TGF-β1 by LY364947 reversed the high expression of ECM in rat BMVSMCs under ANGII stimulation. (B) Deletion of EP3 rescued ANGII-induced activation of TGF-β1/ SMAD2/signaling. (C) The downregulation of ECM by the inhabitation of EP3 was abolished by pretreatment with PTX in BMVSMCs. BMVSMCs, brain microvascular smooth muscle cells; ECM, extracellular matrix; TGF-β1, transforming growth factor-beta1; ANGII, Angiotensin II. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; NS, no significant statistical difference.

Figure 5

(A) Quantitative results of CBF. (B) Representative picture of CBF. CBF, cerebral blood flow; RHRsp, Stroke-prone renovascular hypertensive rat; H, high; L, low; *, $p \leq 0.05$, ***, $p \leq 0.01$.

A**Escape Latency** **B****Time Spent in the Target Quadrant** **C****Number of Times Crossing the Platform** **Figure 6**

(A) Morris water maze test: escape latency in place navigation test. (B) Morris water maze test: time spent in the target quadrant in the spatial probe test. (C) Morris water maze test: the number of times crossing the platform in the spatial probe test. RHRsp, Stroke-prone renovascular hypertensive rat; **, $p \leq 0.01$; ***, $p \leq 0.001$.

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