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RESEARCH PAPER

Integrin β3 mediates cerebrovascular remodelling through Src/ClC-3 volume-regulated Cl^- channel signalling pathway

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BACKGROUND AND PURPOSE

Cerebrovascular remodelling is one of the important risk factors of stroke. The underlying mechanisms are unclear. Integrin β3 and volume-regulated ClC-3 Cl^- channels have recently been implicated as important contributors to vascular cell proliferation. Therefore, we investigated the role of integrin β3 in cerebrovascular remodelling and related Cl^- signalling pathway.

EXPERIMENTAL APPROACH

Cl^- currents were recorded using a patch clamp technique. The expression of integrin β3 in hypertensive animals was examined by Western blot and immunohistochemisty. Immunoprecipitation, cDNA and siRNA transfection were employed to investigate the integrin β3/Src/ClC-3 signalling.

KEY RESULTS

Integrin β3 expression was up-regulated in stroke-prone spontaneously hypertensive rats, 2-kidney 2-clip hypertensive rats and angiotensin II-infused hypertensive mice. Integrin β3 expression was positively correlated with medial cross-sectional area and ClC-3 expression in the basilar artery of 2-kidney 2-clip hypertensive rats. Knockdown of integrin β3 inhibited the proliferation of rat basilar vascular smooth muscle cells induced by angiotensin II. Co-immunoprecipitation and immunofluorescence experiments revealed a physical interaction between integrin β3, Src and ClC-3 protein. The integrin β3/Src/ClC-3 signalling pathway was shown to be involved in the activation of volume-regulated chloride channels induced by both hypo-osmotic stress and angiotensin II. Tyrosine 284 within a concensus Src phosphorylation site was the key point for ClC-3 channel activation. ClC-3 knockout significantly attenuated angiotensin II-induced cerebrovascular remodelling.

CONCLUSIONS AND IMPLICATIONS

Integrin β3 mediates cerebrovascular remodelling during hypertension via Src/ClC-3 signalling pathway.

Abbreviations

2K2C, 2-kidney 2-clip rats; Ang II, angiotensin II; BASMCs, basilar artery smooth muscle cells; CSA, cross-sectional area; DOCA, deoxycorticosterone acetate; L-NAME, N^{ω}-nitro-L-arginine methyl ester; SHRSP, stroke-prone spontaneously hypertensive rats; Tyr, tyrosine; VRAC, volume-regulated chloride channel; VSMCs, vascular smooth muscle cells

Introduction

Cerebrovascular remodelling is a major risk factor for stroke (Ward *et al.*, 2000). Vascular remodelling with structural and functional changes of cellular and noncellular elements has long been confirmed in a variety of animal models of hypertension, such as stroke-prone spontaneously hypertensive rats (SHRSP; Chillon *et al.*, 1996), 2-kidney 1-clip hypertensive rats (Hocher *et al.*, 1999), Deoxycorticosterone acetate (DOCA)-salt hypertensive mice (Du *et al.*, 2008), N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats (Moreau *et al.*, 1995) and angiotensin II (Ang II)-infused hypertensive mice (Ruddy *et al.*, 2009), and experiments in human beings also show that arteries are remodelled in hypertension with a reduction in lumen diameter and increase in media-to-lumen ratio (Heagerty *et al.*, 1993). The latter is an important predictor of cardiovascular events such as myocardial infarction and stroke (De Ciuceis *et al.*, 2007). However, the mechanisms of cerebrovascular remodelling induced by hypertension have not been defined. Although increased haemodynamic stress and hypertrophic stimuli are considered as two main triggers of remodelling (Ward *et al.*, 2000), the downstream signalling is unclear. Interestingly, both mechanical stress and hypertrophic stimuli (Ang II, endothelin-1) activated volume-regulated Cl^- currents in cardiomyocytes and vascular smooth muscle cells (VSMCs; Sato and Koumi, 1998; Shi *et al.*, 2007; Ren *et al.*, 2008). Our previous studies demonstrated that ClC-3 (see Alexander *et al.*, 2013) is responsible for volume-regulated Cl^- currents in VSMCs (Zhou *et al.*, 2005) and the activity of the volume-regulated ClC-3 Cl^- channel displayed a pressure-dependent increase in basilar artery smooth muscle cells (BASMCs) from 2-kidney 2-clip (2K2C) hypertensive rats, and the size of this increase paralleled the severity of cerebrovascular remodelling (Shi *et al.*, 2007). Also, a deficiency of ClC-3 channels attenuated cerebrovascular remodelling during hypertension (Liu *et al.*, 2010; Zheng *et al.*, 2013).

Integrins are transmembrance adhesion proteins that act as mechanotransducers in response to mechanical stresses and convert vascular wall stresses into the intracellular signals (Dahl *et al.*, 2003; Chao and Davis, 2011). Several studies have suggested that integrins played a role in sensing cell volume changes (Pedersen *et al.*, 2001; Dahl *et al.*, 2003) and integrin β1 mediates a stretch-sensitive chloride current in rabbit ventricular myocytes (Browe and Baumgarten, 2003). Other studies have suggested that integrin αVβ3 is required for the proliferation of VSMCs in response to biochemical stimuli (Mawatari *et al.*, 2000; Nguyen Dinh Cat and Touyz, 2011), and is necessary for pressure-induced vascular remodelling (Mawatari *et al.*, 2000; Nguyen Dinh Cat and Touyz, 2011). In this study, we explored the roles of integrin β3 in hypertensive cerebrovascular remodelling and the underlying mechanisms linking this to ClC-3 volume-regulated Cl^- channels.

Methods

Animals

Male SHRSP and Wistar Kyoto rats aged 16 weeks were kindly provided by Dr Su D.F. (Second Military Medical University, Shanghai, China). The animals were kept in ventilated isolator cages under specific pathogen-free (SPF) conditions, air-conditioned animal rooms were maintained at 21°C, humidity 50%, with a 12 h light-dark cycle. Animals had free access to regular chow and sterile water.

2K2C hypertensive rats were prepared as previously described (Zeng *et al.*,1998). Briefly, healthy male Sprague-Dawley rats were anaesthetized by injection of 10% chloral hydrate (3 mg kg^{-1}, i.p.), and ring-shaped silver clips with the internal diameter of 0.3 mm were placed around both right and left renal arteries. The sham-operated rats underwent the same surgical procedure except for the placement of silver clips and served as control. In this and the following procedures the depth of anaesthesia was assessed by absence of hind limb withdrawal in response to paw pinch as well as an absence of a corneal blink reflex.

Ang II-infused hypertensive mice were prepared as previously described (Daugherty *et al.*, 2000). Briefly, 20–25 g mice (C57BL/6J mice, ClC-3$^{+/+}$ wild type (wt) and ClC-3$^{-/-}$ mice) were anaesthetized with pentobarbital (60 mg kg^{-1}, i.p.). Alzet osmotic minipumps (model 1002; ALZA Scientific Products, Mountain View, California, USA) were implanted into the s.c. space of these anaesthetized mice through a small incision in the back of the neck that was closed with surgical glue. Pumps were filled with saline vehicle or solutions of angiotensin II (1.5 mg·kg day^{-1}, 14 day release, Sigma Chemical Co., St. Louis, MO, USA).

All animals used in the study received humane care in compliance with institutional guidelines for the health and care of experimental animals and conformed to the 'Guide for the Care and Use of Laboratory Animals' of the National Institute of Health In China, and were approved by Animal Ethical and Welfare Committee of Sun Yat-sen University (Guangdong, Guangzhou, China). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Immunohistochemistry

Rats were anaesthetized with 10% chloral hydrate and were perfused intracardiacly with 0.1 mol L^{-1} phosphate buffer containing heparin (100 U kg^{-1}) and nitroglycerol (0.3 μg kg^{-1}), followed by 4°C fixative solution containing 4% freshly depolymerized paraformaldehyde in 0.1 mol L^{-1} phosphate buffer for 15 min, the pressure was controlled ≈100 mmHg. The rat brain was carefully removed and sections (8 μm) were prepared from freshly frozen rat basilar arteries as previously described (Shi *et al.*, 2007; Liu *et al.*, 2010)[2]. Randomly selected 3 sections in each group were pretreated with the solution of 3% hydrogen peroxide and methanol at the ratio of 1:50 for 30 min at room temperature, and then blocked with 5% BSA in PBS for 30 min. Then the sections were exposed to α-actin monoclonal antibody (Sigma; dilution 1:400), ClC-3 rabbit polyclonal antibody (Alomone Labs; dilution 1:100) or integrin β3 antibody(1:10050; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight and then were treated with goat FITC-conjugated secondary antibody, goat anti-mouse antibody or goat anti-rabbit antibody. Positive immunostaining was determined with Boster SABC kit(Boster, Wuhan, Hubei, China) and visualized with diaminobenzidine substrate, followed by counterstaining with

haematoxylin (Sigma Chemical Co., St. Louis, Missouri, USA). (Cell Signaling Technology; dilution 1:400) or Alexa Fluor® 488 goat anti-rabbit IgG (Cell Signaling Technology; dilution 1:400) at room temperature for 30 min. The quantification of α-SM-actin staining was analysed using confocal system (OLYMPUS, FV500-IX 81, magnification 400×) and Image-Pro Plus 5.0. For each tested group at each time point, 6 rat brains were taken for the experiment, respectively. A total of 8 randomly selected sections from the area of basilar artery per brain were quantitated for each type of labelling.

Electron microscopy

Electron microscopy were prepared as previously described (Zheng *et al.*, 2013), mice were anaesthetized with 10% chloral hydrate and perfused intracardiacly with 0.1 mol L^{-1} phosphate buffer containing heparin (100 U kg^{-1}) and nitroglycerol (0.3 μg kg^{-1}), followed by 4°C fixative solution containing 4% paraformaldehyde, 0.25% glutaral, 15% saturated trinitrophenol in 0.1 mol L^{-1} phosphate buffer for 15 min. The brains were removed, and the tissue blocks containing the basilar artery at midpoint were cut into cubes of 1 mm × 1 mm × 3 mm, and then immersed into fixative solution at 4°C overnight. After further fixed in 2% osmic acid for 2 h, the tissue blocks were dehydrated via graded alcohols and embedded in Epon 812. Ultrathin sections with thickness of 80–100 nm were prepared and stained with uranyl acetate and lead citrate, and then were viewed under a transmission electron microscope (FEI TECNAI spirit G2, USA).

Immunoprecipitation

Cells were lysed on ice in Nonidet P-40 buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1%Nonidet P-40, Leupeptin (1 μg·mL^{-1}), aprotinin (1 μg·mL^{-1}), and PMSF (400 μM). After clarification, 500 μg of protein were immunoprecipitated with anti-HA (Cell Signaling Technology) or IgG (Cell Signaling Technology) at 4°C overnight, then with 50 μg of Protein G Plus-Agarose beads (Santa Cruz Biotechnology Inc; USA) for 90 min at 4°C. Protein complexes were subjected to Western blotting with the indicated antibodies. For comparison, 40 μg of cell lysate were applied to adjacent lanes.

Electrophysiological experiments

The Cl^- current was recorded with an Axopatch 200B Amplifier (Axon Instruments, Foster City, CA, USA) using amphotericin-perforated patch recording technique. Amphotericin-B was freshly dissolved in DMSO at a concentration of 60 mg·mL^{-1}, and then 20 μL of this solution was mixed with 5 mL of pipette solution by vortexing. The cell was held at −40 mV, and test potentials were applied from −100 mV to +120 mV for 400 ms in +20 mV increments at an interval of 5 s. Currents were filtered at frequency of 2 kHz and digitized at 5 kHz using pCLAMP8.0 software (Axon Instruments).

Details of the methods concerning the cell culture, Western blot analysis, electrophysiological experiments, cell transfection and generation of stable ClC-3 mutant-expressing cell lines are presented in the Supporting Information.

Statistical analysis

All data are expressed as means ± SEM. Statistical analyses were performed using Student's *t*-test or ANOVA followed by a *post hoc* comparison using the least significant difference test. '*n*' represents the number of independent tests. Values of $P < 0.05$ were considered statistically significant.

Results

Integrin β3 was involved in cerebrovascular remodelling in hypertension

In rabbit ventricular myocytes, direct and specific stretch of integrin β1 activated an outwardly rectifying, tamoxifen-sensitive Cl^- current, which resembled the volume-regulated chloride channel (VRAC, the currents, $I_{Cl,vol}$; Browe and Baumgarten, 2003). Meanwhile, other studies suggested that integrin β3 was involved in hypertensive vascular remodelling (Han *et al.*, 2007; Panchatcharam *et al.*, 2010). So, we determined the expression of integrin β1 and β3 in BASMCs. To our surprise, the expression of integrin β1 was almost non-existent in BASMCs, whereas integrin β3 was highly expressed (see Supporting Information Figure S1). In 2K2C hypertensive rats, the BP increased progressively after the operation (Supporting Information Figure S2) and integrin β3 expression was up-regulated significantly in basilar arteries from these rats as shown by Western blot (Figure 1A); this was positively correlated with BP, $r = 0.8112$ (Supporting Information Figure S3a). The increases in integrin β3 expression were further confirmed by immunohistochemical staining of integrin β3 (Supporting Information Figure S4). Our previous studies showed that smooth muscle α-actin staining was increased time-dependently in basilar arteries from 2K2C hypertensive rats. The mean values of medial cross-sectional area (CSA) increased in the hypertensive group after 4 weeks post-operatively compared with those in corresponding control groups (Liu *et al.*, 2010). The present study showed that integrin β3 expression was positively correlated with the medial CSA of the basilar artery from 2K2C hypertensive rats, $r = 0.6025$ (Figure 1D). This increased expression of integrin β3 in basilar arteries during hypertension was further confirmed in two other animal models, SHRSP and Ang II-infused hypertensive mice (Figure 1B and C). Next, we investigated the functional role of integrin β3 in the proliferation of BASMCs. Ang II (200 nM) increased BrdU incorporation in BASMCs by 1.57-fold ($P < 0.05$, $n = 5$). Integrin β3 knockdown by siRNA (see Supporting Information Figure S5) significantly inhibited the proliferation of BASMCs induced by Ang II (Figure 1E).

Our previous study demonstrated that the expression of ClC-3 in basilar arteries was increased gradually with the development of cerebrovascular remodelling induced by hypertension (Liu *et al.*, 2010), and a deficiency of ClC-3 attenuated cerebrovascular remodelling in DOCA-salt hypertension (Zheng *et al.*, 2013). In the present study we showed that integrin β3 expression was positively correlated with ClC-3 expression in basilar arteries from 2K2C hypertensive rats, $r = 0.641$ (see Supporting Information Figure S3b). These findings suggest that integrin β3 is involved in cerebrovascular remodelling induced by hypertension and the

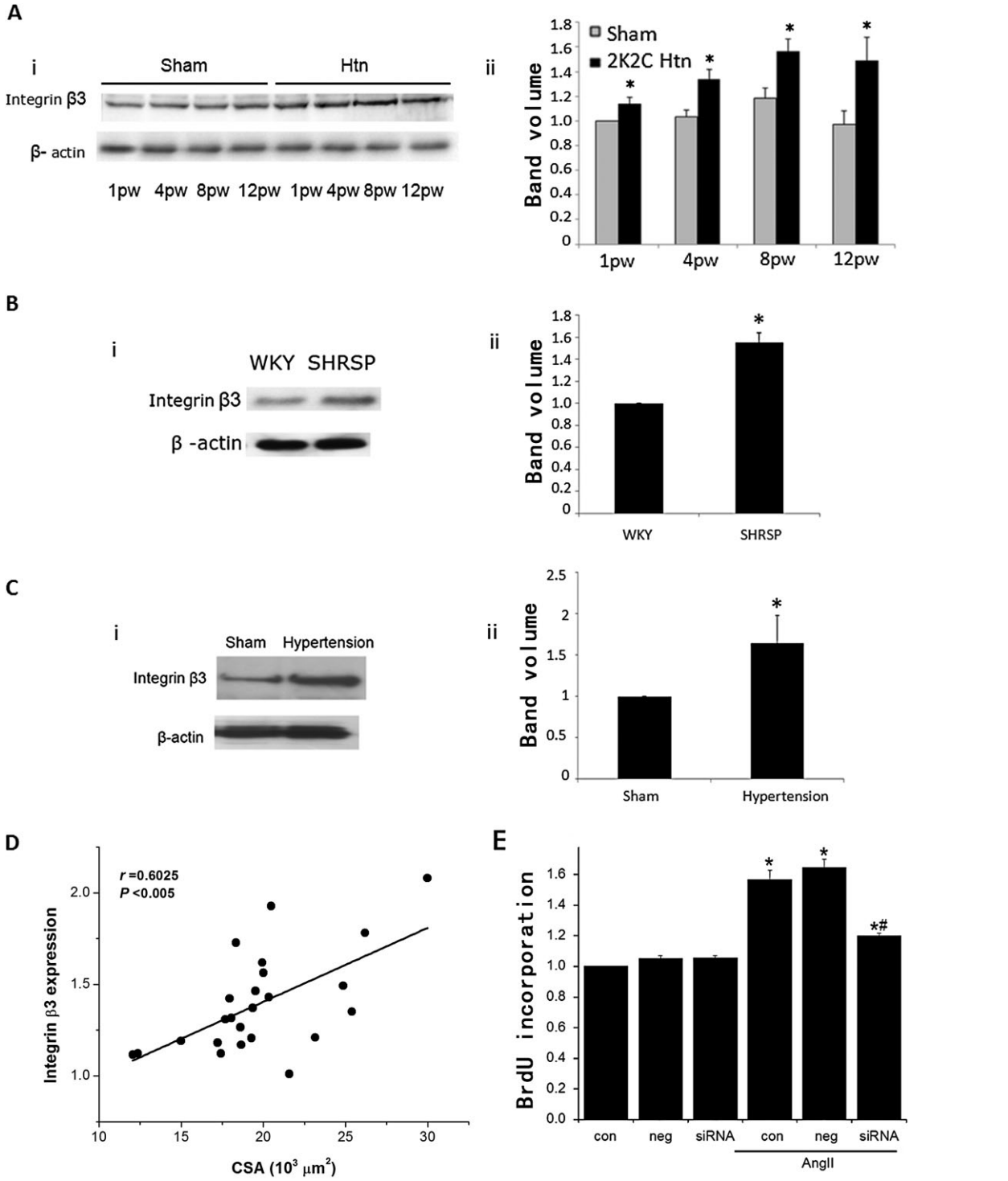


Figure 1

Integrin β3 expression in different hypertensive animal models. (A) (i) Expression of integrin β3 in basilar artery from 2K2C hypertensive groups and corresponding control groups. (ii) Densitometric analysis of integrin β3 expression. Values are mean ± SEM. $n = 6$ from 72 separate rats. (*$P < 0.05$ compared with sham in each group.). (B) (i) Expression of integrin β3 in basilar artery from 16-week-old WKY rats and age-matched SHRSP rats. (ii) Densitometric analysis of integrin β3 expression, $n = 4$, from eight separate rats. (*$P < 0.01$ compared with WKY). (C) (i) Expression of integrin β3 in basilar artery from Ang II hypertensive mice. (ii) Densitometric analysis of integrin β3 expression, $n = 4$, from 20 separate rats. (*$P < 0.01$ compared with sham). (D) Correlative analysis shows that there was a positive correlation between integrin β3 expression and medial CSA of basilar artery from 2K2C hypertensive rats (data from 24 rats and three independent experiments, $r = 0.6025$ $P < 0.005$). (E) Effect of integrin β3 knockdown on Ang II-induced BASMCs proliferation. Increase in cell growth induced by 200 nM Ang II was determined by assaying BrdU incorporation. Incubation of integrin β3 siRNA for 48 h significantly decreased BrdU incorporation ($n = 5$; *$P < 0.05$ vs. control, # $P < 0.05$ vs. control AngII).

volume-regulated ClC-3 chloride channel might be one of the mechanisms underlying this effect.

Integrin β3 mediated VRAC through the Src/ClC-3 signalling pathway in BASMCs

Src kinase activation is required for integrin-mediated osmosensing (Häussinger and Reinehr, 2011). Phosphorylation of tyrosine 416 (Tyr^{416}) is a critical step leading to full activation of Src kinase (Thomas and Brugge, 1997). We determined the role of integrin β3 in Src kinase activation induced by hypotonic solution. Figure 2A shows that hypotonic solution increased phospho-Src (pY416) in a time-dependent manner. The increase in phospho-Src (pY416) induced by hypotonic solution treatment for 30 s was significantly enhanced by integrin β3 overexpression (Figure 2B), but reduced by integrin β3 knockdown (Figure 2C). These results suggest that integrin β3 mediates the Src phosphorylation induced by hypotonic solution.

Previous studies in ventricular myocytes demonstrated that stretch of integrin β1 activates an outwardly rectifying Cl^- current, of which the characteristics resembled the $I_{Cl.vol}$ (Browe and Baumgarten, 2003). Our group and others recently demonstrated cell swelling and pressure-induced membrane stretch stimulated VRAC, and the currents were dependent on ClC-3 expression (Duan *et al.*, 1997; Zhou *et al.*, 2005; Qian *et al.*, 2009). Here, we further investigated the signalling mechanisms linking integrin β3 and the ClC-3 chloride channel.

Hypotonic solution activated $I_{Cl.vol}$ in wild-type (wt) BASMCs with current densities of -13.2 ± 1.4 pA·pF^{-1} at –80 mV and 24.9 ± 6.7 pA·pF^{-1} at +80 mV. Knockdown of integrin β3 by siRNA almost completely blocked the currents, and current densities of $I_{Cl.vol}$ in integrin β3 siRNA-transfected cells were -2.4 ± 3.8 pA·pF^{-1} at –80 mV and 3.7 ± 6.4 pA·pF^{-1} at +80 mV ($n = 6$; Figure 3).

Previous studies demonstrated that Src kinase was required for integrin-mediated VRAC activation (Browe and Baumgarten, 2003; Barfod *et al.*, 2005). We previously found that the Src kinase inhibitor SU6656 inhibited $I_{Cl.vol}$ in a concentration-dependent manner and Src kinase regulated $I_{Cl.vol}$ by interacting with ClC-3 protein and phosphorylating the channel at tyrosine 284 (Tyr^{284}). The phosphomimetic mutation, Y284D, significantly increased ClC-3-mediated Cl^- current and Cl^- efflux, while the non-phosphorylating mutation, Y284F, almost completely abolished the ClC-3-mediated

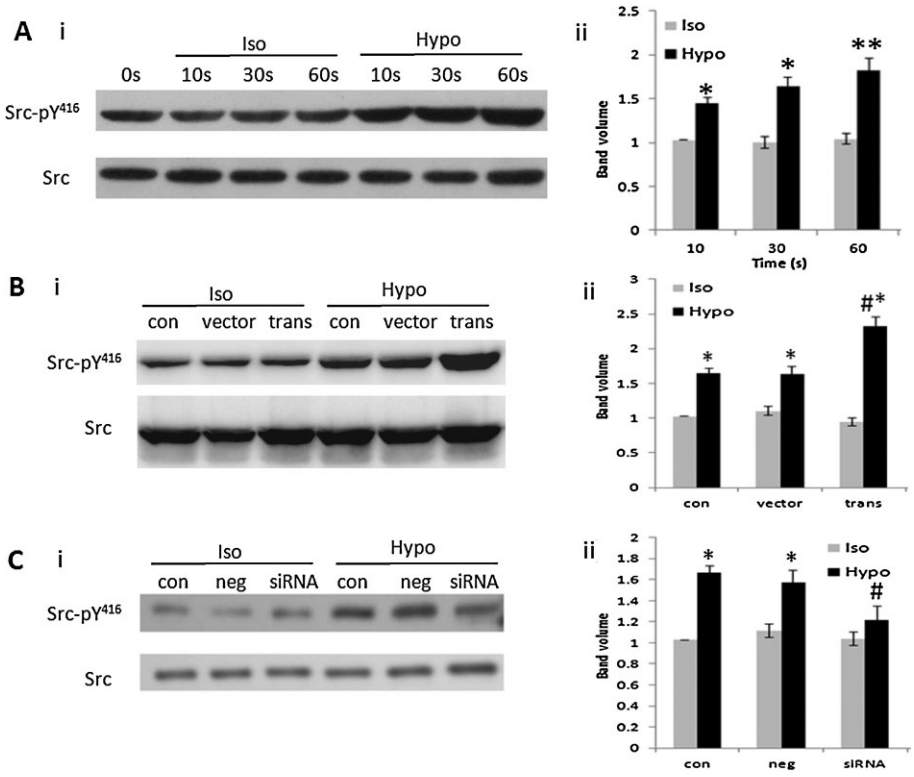


Figure 2

Integrin β3 mediated Src phosphorylation induced by hypotonic solution. (A) Phospho-Src (pY^{416}) stimulated by hypotonic solution from 0 to 60 s. (i) Representative Western blot is shown. (ii) Densitometric analysis shows that phospho-Src (pY^{416}) induced by hypotonic solution was time dependent (*$P < 0.05$ vs. isotonic (Iso) group, **$P < 0.01$ vs. Iso group; data from five different experiments). (B) Effect of integrin β3 cDNA transfection on phospho-Src (pY^{416}). (i) Representative Western blot. (ii) Densitometric analysis. (*$P < 0.05$ vs. Iso group, #$P < 0.05$ vs. vector group; data from five different experiments). (C) Effect of integrin β3 siRNA transfection on phospho-Src (pY^{416}) induced by hypotonic solution. (i) Representative Western blot. (ii) Densitometric analysis. (*$P < 0.05$ vs. Iso group, #$P < 0.05$ vs. negative group; data from five different experiments). Iso, isotonic solution; Hypo, hypotonic solution.

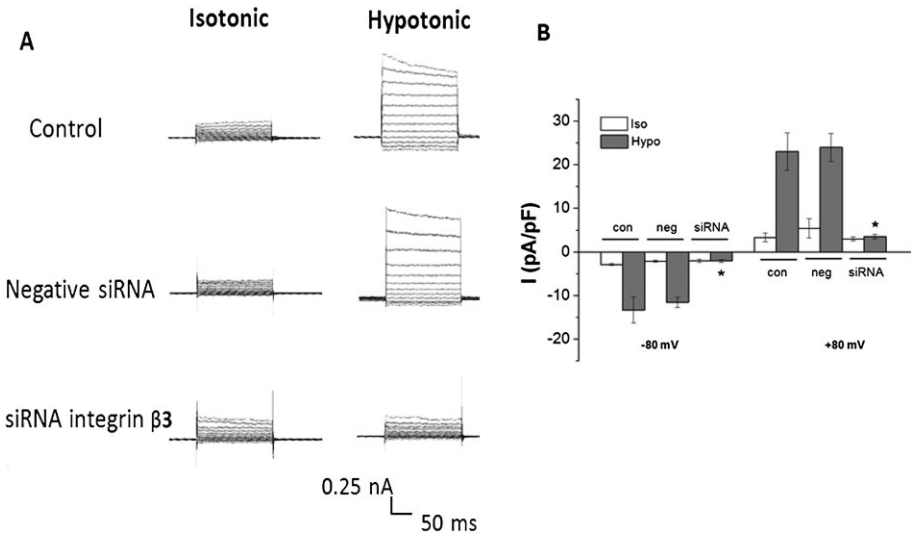


Figure 3

Effect of integrin β3 siRNA transfection on $I_{Cl.vol}$ in BASMCs. (A) Representative traces of Cl^- currents induced by isotonic and hypotonic solution in control, negative siRNA-transfected group and integrin β3 siRNA-transfected groups. (B) Mean current densities measured at −80 mV (downward bars) and +80 mV (upward bars). The cells were transfected for 48 to 72 h (*$P < 0.05$ vs.corresponding control group, $n = 6$). Iso, isotonic solution; Hypo, hypotonic solution.

increased Cl^- current and Cl^- efflux induced by hypotonic solution (Wang *et al.*, 2013). We hypothesized that integrin β3 mediates $I_{Cl.vol}$ through Src and ClC-3. We firstly tested the interaction of Src with integrin β3 and ClC-3 by using co-immunoprecipitation. We found that immunoprecipitation of HA-Src coprecipitated integrin β3 and ClC-3, and immunoprecipitation of ClC-3 coprecipitated integrin β3 and HA-Src (Figure 4A).

Next, we examined the role of integrin β3 in $I_{Cl.vol}$ by using A10 cells stably expressing ClC-3 mutant proteins, Y284F or Y284D. The current densities of $I_{Cl.vol}$ inY284D mutant cells were −11.1 ± 3.7 pA·pF^{-1} at −80 mV and 25.5 ± 7.3 pA·pF^{-1} at +80 mV in isotonic solution, which were significantly enhanced to −61.8 ± 8.8 pA·pF^{-1} at −80 mV and 111.4 ± 17.5 pA·pF^{-1} at +80 mV ($n = 6$, $P < 0.05$) by hypotonic solution. Knockdown of integrin β3 by siRNA did not alter these currents (Figure 4B). In contrast, in Y284F mutant cells hypotonic solution only induced a small $I_{Cl.vol,}$ and overexpression of integrin β3 had little effect on the currents mediated by the Y284F mutant (Figure 4C). These results suggest that integrin β3 mediates $I_{Cl.vol}$ through Src kinase-induced Tyr^{284} phosphorylation in the ClC-3.

Integrin β3 mediates Ang II-induced $I_{Cl.AngII}$ through the Src/ClC-3 signalling pathway

It has been demonstrated that Ang II enhances integrin β3 expression (Brassard *et al.*, 2006), which is consistent with our present results *in vivo* and *in vitro* (Figure 1C and see Supporting Information Figure S6). Previous studies found that Ang II activated outwardly rectifying Cl^- currents ($I_{Cl.AngII}$) in rabbit cardiac myocytes (Ren *et al.*, 2008) and Src TK played an important role in Ang II-initiated signalling transduction (Haendeler and Berk, 2000). We further investigated the effect of integrin β3/Src/ClC-3 signalling on $I_{Cl.AngII}$. In BASMCs Ang II (200 nM) activated an outwardly rectifying Cl^- current ($I_{Cl.AngII}$) in isotonic solution, which was inhibited by hypertonic solution, and knockdown of integrin β3 significantly inhibited this $I_{Cl.AngII}$ (Figure 5A).The $I_{Cl.AngII}$ is dependent on Tyr^{284} phosphorylation of ClC-3 induced by Src kinase, as the current was almost completely inhibited by in the Y284F mutation but significantly potentiated in the Y284D mutation (Figure 5B, $n = 6$, $P < 0.05$). Interestingly, knockdown of integrin β3 did not altered the $I_{Cl.AngII}$ mediated by the Y284D mutant (31.1 ± 3.3 pA·pF^{-1} at +80 mV), and overexpression of integrin β3 did not activate an $I_{Cl.AngII}$ in Y284F (Figure 5B). These results reveal that the $I_{Cl.AngII}$ is mediated through the integrin β3/Src/ClC-3 signalling pathway.

ClC-3 knockout attenuated cerebrovascular remodelling in Ang II hypertension

We further determined the functional role of the integrin β3/Src/ClC-3 signalling pathway in the development of cerebrovascular remodelling using ClC-3 knockout (ClC-3$^{-/-}$) mice treated with Ang II. As indicated by smooth muscle α-actin immunocytochemical staining, ClC-3 knockout ameliorated the morphological changes in mice cerebral basilar artery that occur during hypertension (Figure 5C, i). Also Ang II treatment led to a significant increase in media thickness and decrease in the diameter of the internal lumen, resulting in an increased media-to-lumen ratio in basilar arteries from wt mice compared with those of control. Medial CSA was also significantly enhanced in AngII-treated wt mice. In contrast, cerebrovascular remodelling was significantly attenuated in the ClC-3$^{-/-}$ mice treated with AngII (Figure 5C, ii). The effects of ClC-3 knockdown on cerebrovascular remodelling were further confirmed by electron microscopy (see Supporting Information Figure S7).

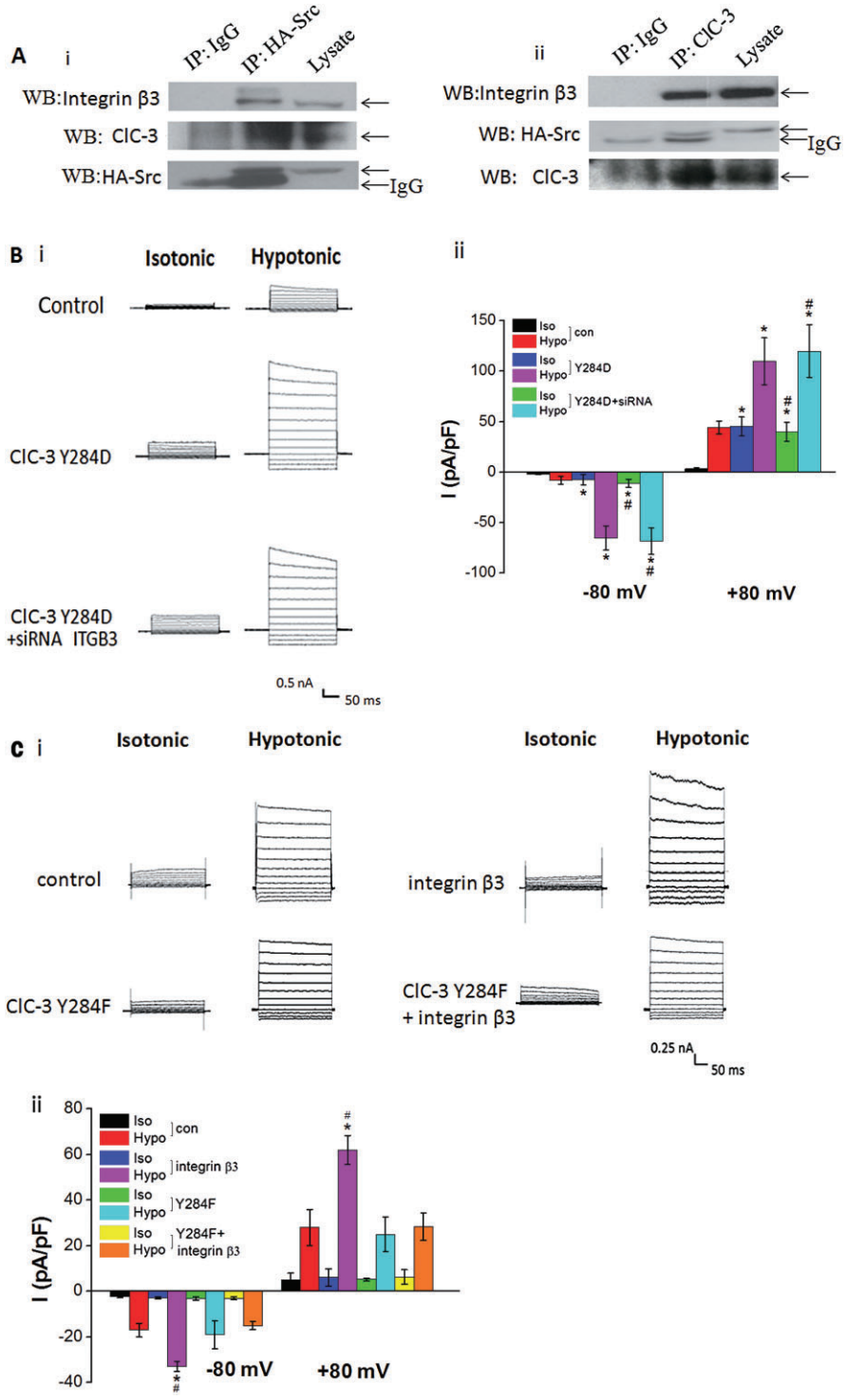


Figure 4

Effect of integrin β3 on $I_{Cl.vol}$ in ClC-3 mutant (Y284D and Y284F) cells. (A) Immunoprecipitation of HA-Src, integrin β3 and ClC-3. (i) Immunoprecipitation of HA-Src coprecipitated with integrin β3 and ClC-3. Protein lysate from HA-SrcA BASMCs were immunoprecipated with HA-specific antibody or control IgG. The immunoprecipitations were blotted with anti-integrin β3, anti-ClC-3 or anti-HA-Src. (ii) Immunoprecipitation of ClC-3 coprecipitated with integrin β3 and HA-Src. Protein lysate from BASMCs were immunoprecipated with anti-ClC-3 antibody or control IgG and blotted with anti-integrin β3, anti-HA-Src, or anti-ClC-3 ($n = 4$). (B) Integrin β3 siRNA did not alter the $I_{Cl.vol}$ in A10 cells stably overexpressing the ClC-3 Y284D mutant. (i) Representative traces of Cl^- current. (ii) Mean current densities measured at –80 mv (downward bars) and+80 mv (upward bars; $^*P < 0.05$ vs. corresponding control group, $\#P > 0.05$ vs. Y284D group, $n = 5$). (C) Transfection of integrin β3 cDNA significantly increased the $I_{Cl.vol}$ in A10 cells, but had no effect on the $I_{Cl.vol}$ in ClC-3 Y284F mutant cells. (i) Representative traces of Cl^- current. (ii) Mean current densities measured at –80 mV (downward bars) and +80 mV (upward bars) ($^*P < 0.05$ vs. control group, $\#P > 0.05$ vs. Y284F group, $n = 5$).

Discussion and conclusions

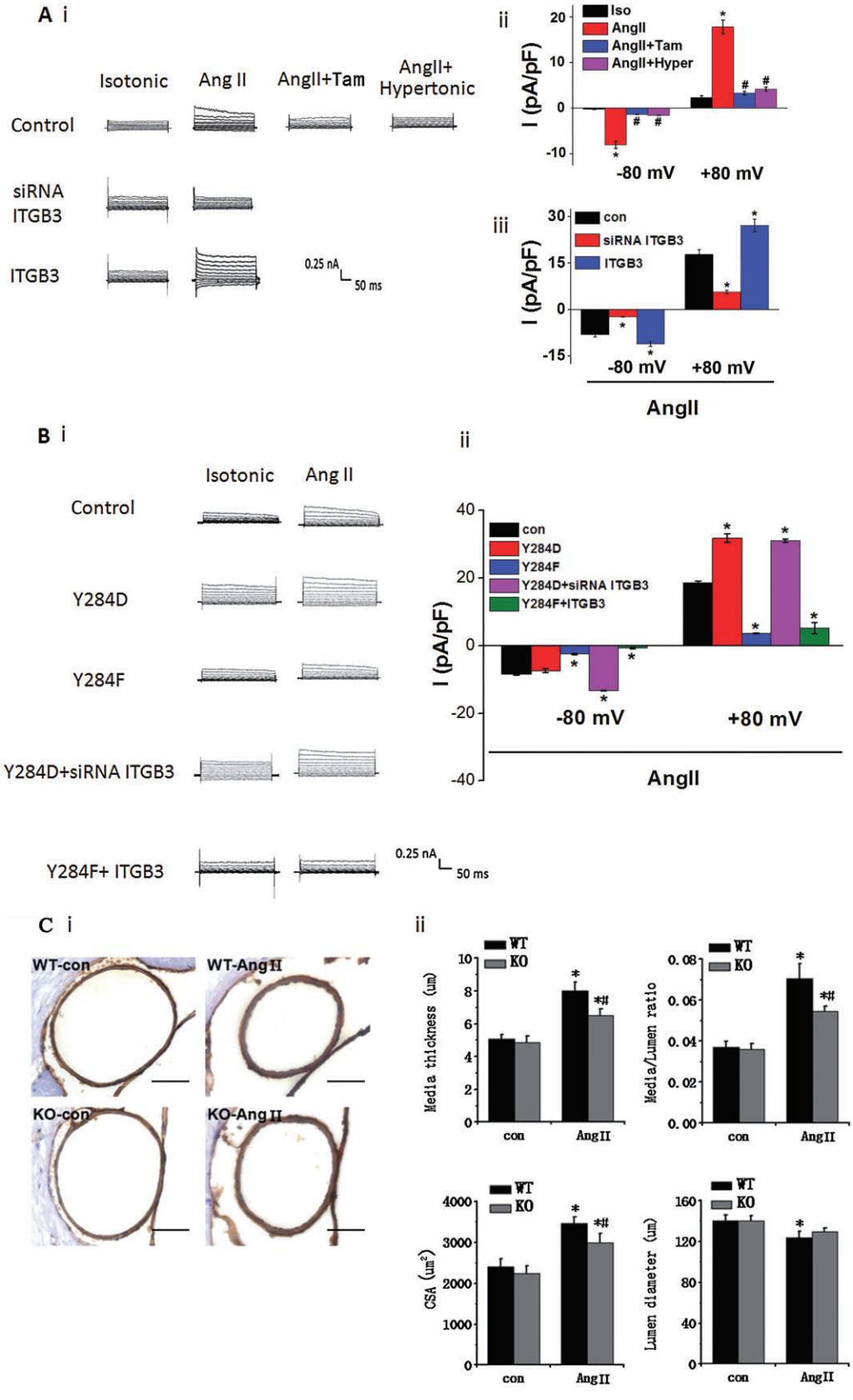
Recent evidence obtained by us and others has demonstrated that the ClC-3 chloride channel plays an important role in the vascular remodelling that occurs during hypertension (Dai *et al.*, 2005; Guan *et al.*, 2006; Zheng *et al.*, 2013), by accelerating the proliferation of VSMCs (Wang *et al.*, 2002; Tang *et al.*, 2008) and preventing their apoptosis (Okada *et al.*, 2006; Qian *et al.*, 2011). We previously found that ClC-3 channel activity and protein expression were significantly increased in BASMCs from 2K2C hypertensive rats and these increases paralleled the severity of cerebrovascular remodelling (Shi *et al.*, 2007). Moreover, simvastatin ameliorated hypertension-induced cerebrovascular remodelling by inhibiting the proliferation of cerebrovascular smooth muscle cell and suppressing ClC-3 chloride channel activity (Liu *et al.*, 2010). Furthermore, ClC-3 knockout attenuated the cerebrovascular remodelling that occurs in DOCA-salt hypertensive mice (Zheng *et al.*, 2013).

Although the functional roles of the ClC-3 chloride channel in VSMCs have been extensively characterized, the molecular signalling that is associated with ClC-3 channel activation has not been clarified. Recently, we found that Tyr^{284} phosphorylation of the ClC-3 protein targeted by Src kinase was required for ClC-3 channel activation; the $I_{Cl.vol}$ was completely blocked in the non-phosphorylatable mutation Y284F, whereas this Cl^- current mediated by ClC-3 was increased in the phosphomimetic mutation Y284D (Wang *et al.*, 2013). Furthermore, there is accumulating evidence supporting the notion that Y284 phosphorylation of ClC-3 plays an important role in cell apoptosis (Dupere-Minier *et al.*, 2004; Wang *et al.*, 2013). However, the upstream and downstream components of this Src/ClC-3 signalling pathway remain to be identified. In this study, we demonstrated that integrin β3 acts as an upstream component of the Src/ClC-3 signalling pathway; integrin β3 siRNA and cDNA transfection inhibited and enhanced the $I_{Cl.vol}$ mediated by ClC-3, respectively, but had no effect in ClC-3 Y284D or Y284F mutant cells.

Integrins are cell surface adhesion proteins that mediate a variety of physiological functions and pathological processes and consist of heterodimers composed of an α-chain and a β-chain. In mammals, 18 α- and 8 β-subunits have been characterized (Barczyk *et al.*, 2010). We focused our study on β-subunits because of previous reports suggesting that activation of integrin β1 induced VRAC (Browe and Baumgarten, 2003). It should be noted that integrin β1 also mediated VRAC via Src in rabbit ventricular myocytes, indicating that our findings showing the involvement of a integrin/Src/ClC-3 signalling pathway may not be unique to VSMCs. The finding that different integrin β-subunits can mediate the Src/ClC-3 signalling pathway may be because the integrins expressed by different cell types vary greatly.

Hypertrophic stimuli, such as Ang II, and mechanical stress on VSMCs are important contributors to vascular remodelling during hypertension (Dzau and Gibbons, 1993; Lehoux and Tedgui, 1998). Integrin-mediated stretch-sensitive Cl^- currents have been extensively studied (Browe and Baumgarten, 2003; 2006); however, it is unclear whether integrin-mediated signalling is involved in Ang II-activated Cl^- currents. In the present investigation, we demonstrated that integrin β3/Src/ClC-3 signalling pathway mediated the volume-regulated Cl^- current and Ang II-activated Cl^- current, which resemble mechanical stress and hypertrophic stimuli respectively. To our knowledge, this is the first direct evidence that the integrin β3/Src/ClC-3 signalling pathway mediates the Cl^- current induced by both hypertrophic stimuli (e.g. Ang II) and osmotic stimuli.

Furthermore, the present results obtained in hypertensive animal models *in vivo* suggest that integrin β3 mediated the cerebrovascular remodelling. The expressions of integrin β3 in the basilar artery were up-regulated in the different hypertensive animal models, which included an essential hypertensive (primary hypertensive) model of SHRSP, renovascular hypertensive (secondary hypertensive) model of 2K2C hypertensive rats and Ang II-infused hypertensive mice (Ang II-dependent hypertensive model). The increase in integrin β3 expression in the basilar artery from the 2K2C hypertensive rat was positively correlated with the increased BP, and with the hypertension-induced alteration in CSA. Notably, integrin β3 expression was increased 1 week after the operation, when there were no differences apparent between the basilar arteries of hypertensive mice and sham-operated controls. Furthermore, the *in vitro* results showed that knockdown of integrin β3 significantly inhibited Ang II-induced proliferation of the BASMCs. These results suggest that integrin β3 mediated the cerebrovascular remodelling during hypertension. We have noted the report that an up-regulation of αvβ3 integrin had a crucial role in the mesenteric artery of TGR (mRen2)27 (model of hypertension) during the eutrophic remodelling process (Heerkens *et al.*, 2006). This report suggested that the αv subunit of αvβ3 integrin, but not the β3 subunit, played a key role in the eutrophic remodelling of resistance arteries. One possible explanation for this inconsistency is that vascular remodelling may be different among vessel types and models of hypertension. Consistent with our previous studies in 2K2C hypertensive rats (Shi *et al.*, 2007; Liu *et al.*, 2010; Wang *et al.*, 2012), cerebral vessels from Ang II-infused hypertensive mice in the present study underwent the hypertrophic remodelling process, which is characterized by increases in blood vessel wall thickness, outer blood vessel wall diameter, CSA and a reduction in inner blood vessel wall diameter (Guan *et al.*, 2006; Shi *et al.*, 2007; Liu *et al.*, 2010). This is different from he αv-mediated eutrophic remodelling, which is characterized by a reduction in lumen diameter and unchanged CSA (Sonoyama *et al.*, 2007). The signs of vascular remodelling are not uniform across vessel types or animal models, for example, pial arteries with smaller lumens and thicker walls were found in SHRSP (Chillon *et al.*, 1996), L-NAME-induced hypertensive rats (Kitamoto *et al.*, 2000), DOCA-salt hypertensive mice (Liu *et al.*, 2010; Zheng *et al.*, 2013); however, in 1-kindey 1-clip hypertensive rats, pial arterioles displayed thicker walls but normal lumens (Baumbach and Hajdu, 1993). It is noteworthy that these studies (Baumbach and Hajdu, 1993; Chillon *et al.*, 1996; Kitamoto *et al.*, 2000; Liu *et al.*, 2010; Zheng *et al.*, 2013) indicate that BP alone is not the sole stimulus for vascular remodelling. Interestingly, we recently demonstrated that in DOCA-salt hypertensive mice, propranolol, which reduced BP effectively, failed to prevent basilar artery from remodelling (Liu *et al.*, 2010; Zheng *et al.*, 2013). As integrin/Src/ClC-3 signalling is the common


A i
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Ang II
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Media thickness (um)
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Figure 5

Involvement of integrin β3 in $I_{Cl.AngII}$ and ClC-3 knockout attenuates cerebrovascular remodelling in Ang II-infused hypertensive mice. (A) In BASMCs, Ang II (200 nM) induced an outward rectifying current Cl^- current ($I_{Cl.AngII}$) in isotonic solution, which was completely inhibited in hypertonic solution (Hyper) and by tamoxifen (Tam, 10 μm), indicating that this current was a volume-regulated Cl^- current. $I_{Cl.AngII}$ was also completely blocked by integrin β3 siRNA (siRNA ITGB3) and significantly enhanced by integrin β3 cDNA(ITGB3). (i) Representative traces of $I_{Cl.AngII}$. (ii) Mean current densities of control group with different treatment measured at −80 mV (downward bars) and +80 mV (upward bars). (iii) Mean current densities of angiotensin II-treated control, siRNA ITGB3 and ITGB3 groups at −80 mV (downward bars) and +80 Mv (upward bars; $*P <$ 0.05 vs. control group, # $P < 0.05$ vs. Ang II group, $n = 5$). (B) $I_{Cl.AngII}$ was regulated by Tyr^{284} phosphorylation in ClC-3. In ClC-3 Y284D and Y284F cells, $I_{Cl.AngII}$ was enhanced and inhibited respectively. Integrin β3 cDNA transfection did not alter Y284F effect on $I_{Cl.AngII.}$ Similarly, integrin β3 siRNA transfection did not reverse Y284D effect on $I_{Cl.AngII}$. (i) Representative traces of Cl^- current. (ii) Mean current densities measured at −80 mV (downward bars) and +80 mV (upward bars) ($*P < 0.05$ vs. control group, $n \geq 6$). (C) ClC-3 knockout ($ClC\text{-}3^{-/-}$) ameliorated the morphological changes of mice cerebral basilar artery occurring during hypertension. (i) wt ($ClC\text{-}3^{+/+}$) mice and $ClC\text{-}3^{-/-}$ mice were treated with or without Ang II. Vascular media smooth muscle was specifically stained as brown with α-actin antibody. Scale bars, 50 μm. Representative pictures are shown. (ii) Statistical analysis of media thickness, internal lumen diameter, media-to-lumen ratio and medial CSA ($n = 6$ per group, $*P < 0.05$, Ang II mice vs.control mice; $\#P < 0.05$, wt-AngII mice vs. $ClC\text{-}3^{-/-}$-AngII mice).

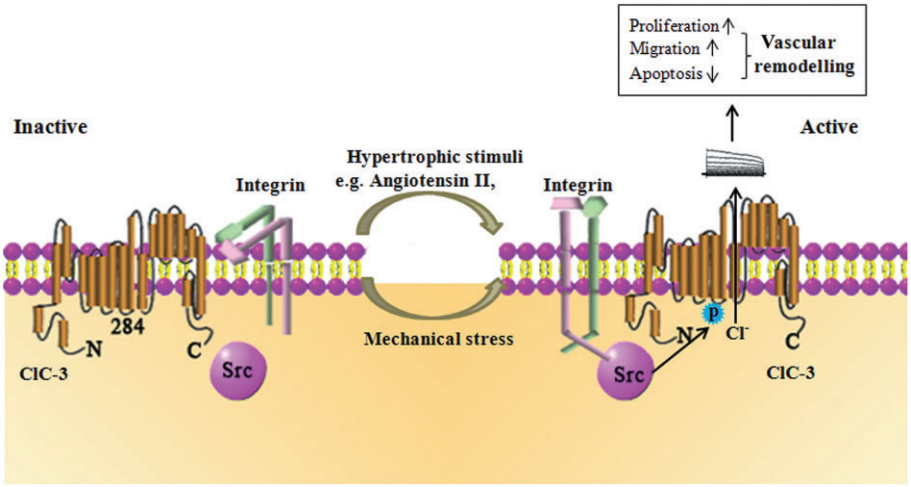

Figure 6

Integrin β3/Src/ClC-3 signalling pathway. Hypertrophic stimuli (such as Ang II), mechanical and osmotic stimuli could be sensed by integrin β3, leading to Src phosphorylation of ClC-3 at Tyr^{284}, triggering activation of the ClC-3 chloride channel and efflux of Cl^-. The ClC-3 chloride channel is associated with cerebrovascular remodelling, by promoting the proliferation and migration of VSMCs and preventing their apoptosis.

pathway in haemodynamic stress and hypertrophic stimuli-initiated pro-remodelling signalling, they may serve as more efficacious therapeutic candidates.

There is accumulating evidence supporting the important roles of ClC-3 in proliferation (Wang *et al.*, 2002; Guan *et al.*, 2006; Tang *et al.*, 2008), apoptosis (Guan *et al.*, 2006; Okada *et al.*, 2006; Qian *et al.*, 2011) and migration (Ganapathi *et al.*, 2013) in VSMCs, making ClC-3 a novel therapeutic target against vascular remodelling. We recently found that knockout of ClC-3 *in vivo* attenuated the disorganization of VSMCs and extracellular matrix accumulation in DOCA-salt hypertension (Liu *et al.*, 2010; Zheng *et al.*, 2013). In addition to the influence of MMPs/ TIMP expression and TGF-b1/Smad3 signalling, the effects of ClC-3 on Akt (Tang *et al.*, 2008), NF-κB (Miller *et al.*, 2007) and redox (Miller *et al.*, 2007; Matsuda *et al.*, 2010) signalling are possible mechanisms linking ClC-3 to vascular remodelling.

Taken together, the results of the present study demonstrated that integrin β3/Src/ClC-3 signalling is involved in cerebrovascular remodelling in both a primary and secondary hypertension model. Mechanical strain and hypertrophic stimuli are important pathogenic factors in hypertensive vascular remodelling. We found that the integrin β3/Src/ClC-3 signalling pathway was involved both in hypo-osmotic stress (mimicking mechanical strain) and Ang II (main hypertrophic stimuli)-induced VRAC activation, triggering a series of mitogenic and anti-apoptotic signalling resulting in increased vascular cell proliferation and migration, and reduced apoptosis, finally leading to cerebrovascular remodelling (Figure 6).

Acknowledgements

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12654

Figure S1 Integrin β1 and integrin β3 expression in BASMCs. Integrin β3 was detected while integrin β1 was hardly detected in BASMCs. Human umbilical vein endothelial cells (HUVECs) were used as positive control for integrin β1 expression. $n = 8$.

Figure S2 Blood pressure of hypertensive animals. (a) Blood pressure of 2K2C hypertensive group and sham group at different time point. PW, postoperative week. $^*P < 0.01$ compared with sham in each group; $\#P < 0.01$ compared with 1PW in each group, $n = 6$. (b) Blood pressure of SHRSP rats and WKY rat at 16 weeks, $\#P < 0.005$, $n \geq 6$. (c) Blood pressure of angiotensin II infusion induced hypertensive C57BL/6J mice, $n = 5$. Values are mean ± SEM.

Figure S3 (a) Integrin β3 (ITGB3) expression was positively correlated with the blood pressure, correlation coefficient was 0.8125 ($n = 36$ rats from six independent experiments, $P < 0.001$). (b) Integrin β3 expression was positively correlated with ClC-3 during hypertension (24 rats from three independent experiments, $P < 0.005$).

Figure S4 Changes in integrin β3 expression of basilar arteries in 2-kidney, 2-clip hypertensive rats and sham group rats (scale bars, 20 μm). Representative sections were shown, $n = 6$ per group.

Figure S5 The effect of integrin β3 siRNA (ITGB3 siRNA) transfection in BASMCs. BASMCs were transfected with 200 nM integrin β3 siRNA and assayed with reverse transcriptase-PCR (RT-PCR) 48 h later. $^*P < 0.05$ versus negative group, $n = 5$. Values are mean ± SEM.

Figure S6 Densitometric analysis of integrin β3 expression induced by 1 nM, 10 nM, 100 nM and 1000 nM angiotensin II, the solvent of angiotensin II was dimethyl sulfoxide (DMSO, 0.1%; $n = 5$; $^*P < 0.05$ vs. control).

Figure S7 Electron microscopy images of cerebral basilar arteries from wt and ClC-3$^{-/-}$ mice with or without angiotensin II implantation. Angiotensin II resulted in smooth muscle cell disarrangement (◊), intercellular space enlargement (→) and vacuolization (Δ) in wt mice; while in the basilar artery of ClC-3$^{-/-}$ mice, these ultrastructural changes did not occur. Black bar represents 1 μm. Six mice were studied in each group.