Simvastatin Ameliorates Rat Cerebrovascular Remodeling During Hypertension via Inhibition of Volume-Regulated Chloride Channel

Yu-Jie Liu, Xiao-Guang Wang, Yong-Bo Tang, Jing-Hui Chen, Xiao-Fei Lv, Jia-Guo Zhou, Yong-Yuan Guan

Abstract—Statins have pleiotropic actions against the development of vascular remodeling and the incidence of ischemic stroke. Although previous studies have suggested that posttranslational modification of several proteins, such as Rho by mevalonate-derived isoprene groups, geranylgeranyl pyrophosphate or farnesyl pyrophosphate, underlie the pleiotropic effects of statins, the detailed mechanisms remain elusive. Recent growing evidence demonstrated that CIC-3 volume-regulated chloride channel plays an important role in cell proliferation, and the activity of this channel is increased in basilar smooth muscle cells from a hypertensive rat. We hypothesized that inhibition of volume-regulated chloride channel may contribute to the beneficial effects of statins on cerebrovascular remodeling during hypertension. Our study here demonstrated that simvastatin ameliorated hypertension-caused cerebrovascular remodeling. In rat basilar smooth muscle cells, simvastatin inhibited cell proliferation and activation of volume-regulated chloride channel, and these effects of simvastatin were abolished by pretreatment with mevalonate or geranylgeranyl pyrophosphate. In addition, Rho A inhibitor C3 exoenzyme and Rho kinase inhibitor Y-27632 both reduced cell proliferation and activation of volume-regulated chloride channel. Moreover, CIC-3 overexpression decreased the suppressive effect of simvastatin on cell proliferation and increased estimated IC_50 of simvastatin on endothelin-1- and hypo-osmolarity-induced cell proliferation from 3.40 ± 0.08 and 3.50 ± 0.10 μmol/L to 5.30 ± 0.70 and 5.60 ± 0.70 μmol/L, respectively (P < 0.01; n = 6). Furthermore, the expression of CIC-3 was increased in basilar artery during hypertension, and simvastatin normalized the upregulation of CIC-3. Our data suggested that simvastatin ameliorates cerebrovascular remodeling in the hypertensive rat through inhibition of vascular smooth muscle cell proliferation by suppression of volume-regulated chloride channel. (Hypertension. 2010;56:445-452.)

Key Words: volume-regulated chloride channel • simvastatin • hypertension • cerebrovascular remodeling • basilar smooth muscle cell

Cerebral arterioles undergo remodeling of the vascular walls during chronic hypertension, which is caused by the coordination of vascular smooth muscle cell (SMC) proliferation and migration, endothelial cell dysfunction, inflammation, and fibrosis.1–3 It has been generally accepted that vascular remodeling is an important determinant of increased risk of stroke that accompanies chronic hypertension.4,5 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are extensively used in clinic to treat hypercholesterolemia by inhibiting cholesterol biosynthesis. A number of large clinical trials and animal experiments have established that statins are available to reduce the mortality and morbidity of cardiovascular events, such as coronary artery disease and stroke.6–9 These beneficial effects of statins cannot be merely attributed to their lipid-lowering property.8,10 Recent growing evidence has demonstrated that statins have pleiotropic effects, including inhibition of vascular SMC proliferation, improvement of endothelial function, increase of NO availability, reduction of inflammatory response, and oxidative stress production.8,11,12 It has been reported that these beneficial effects of statins are associated with the mevalonate (MVA)-derived isoprenoid intermediates, geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), which serve as lipid attachments for the posttranscriptional modification of several proteins, such as Ras, Rho, and Rac, to their active GTP-binding state.10–13 However, the detailed mechanisms by which statins exert these effects remain to be elucidated. It has been reported that statins could regulate the activities of a variety of ion channels, such as 1-type calcium channel,14 calcium-activated potassium chan-

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nel,15 and nonselective cation channel,16 in the vascular SMC and endothelial cell.

Volume-regulated chloride channel (VRCC) has been documented to play an important role in the regulation of cell volume, proliferation, differentiation, and apoptosis.17–21 Although the molecular identity of VRCC is still under debate, our previous work demonstrated that ClC-3, a member of the voltage-gated ClC chloride channel family, is the key component of VRCC in vascular SMCs.22 Moreover, our recent study found that the activity of VRCC is increased in hypertensive rat basilar SMCs (BASMCs), and its increment parallels the severity of the cerebrovascular remodeling,23 suggesting that VRCC is involved in vascular remodeling process during chronic hypertension.

The aim of our present study, therefore, was to investigate the effects of simvastatin on the cerebrovascular remodeling in the 2-kidney 2-clip hypertension model. Furthermore, we evaluated the functional role of VRCC in the beneficial effects of simvastatin on rat cerebrovascular remodeling and BASMC proliferation.

Materials and Methods

An expanded Material and Methods is in the online Data Supplement, available at http://hyper.ahajournals.org.

All of the animal experimental procedures were performed in accordance with the policies of the Animal Care and Use Committee, Sun Yat-Sen University, and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China. Four- to 5-week-old male Sprague-Dawley rats (body weight: 90 to 120 g) were anesthetized by injection of 10% chloral hydrate (3 mg/kg IP), and 2-kidney 2-clip renovascular hypertensive rats were operated as described previously.23 Rats were divided into 3 groups, which were the sham, hypertension, and hypertension + simvastatin groups. Simvastatin was dissolved in water and orally administered by gavage at a daily dose of 10 mg/kg after operation. Blood pressure (BP) was measured in conscious rats by tail-cuff plethysmography. Specimens of rat basilar arteries were cut for histochemistry, immunohistochemistry, and electron microscopy studies. Total proteins were extracted from the rat basilar arteries, and the expression of ClC-3 was measured with Western blot. Rat BASMCs were isolated and cultured from rat basilar arteries as described previously.20 These cells were used to study the cell proliferation and to measure intracellular Cl concentration ([Cl]i) and transmembrane Cl currents, as described previously.22,23

All of the data were expressed as mean±SEM. Statistical analyses were performed using a Student t test or ANOVA. Values of P<0.05 were considered significant.

Results

Effects on BP

Consistent with our previous study in a 2-kidney 2-clip hypertensive rat model,24 the BP in the hypertensive group
Increased progressively after operation. Pretreatment with simvastatin mildly decreased the elevated BP from 8 weeks after operation. However, the BPs in the simvastatin-treated group were not significantly different from those in the hypertension group at 1 and 4 weeks after operation (Table).

**Simvastatin Prevented Hypertension-Induced Remodeling of Rat Basilar Artery**

Cross-section histopathologic studies showed that the α-actin staining time-dependently increased in basilar arteries from hypertensive rats (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). The lumen diameter decreased and the wall:lumen area ratio and the mean values of medial cross-sectional area increased in the hypertension group after 4 weeks postoperatively compared with those in the corresponding control groups. The wall diameter did not change until 8 weeks after operation. These changes indicated that the cerebral basilar artery from the hypertensive group underwent remodeling. Simvastatin treatment restored these phenotypes of hypertension-induced basilar artery remodeling from 4 weeks after operation (Figures 1 and S1).

Electron microscopic observation demonstrated that the medial layer of the basilar artery in the hypertension group began to undergo remodeling from 4 weeks after operation, exhibiting migration and proliferation of SMCs, and increased accumulation of collagen fibers. These pathological changes were aggravated as hypertension was developing. From week 8, disarrangement of SMCs, migration of SMCs into the subendothelial region of intima, and accumulation of dense collagen fibers in the intercellular space were observed.

Necrosis of SMCs and endothelial cells, mitochondrial degeneration, and endoplasmic reticulum vacuolization were also shown in our studies. The above ultrastructural remodeling during hypertension was significantly attenuated by simvastatin (Figure S2).

**Simvastatin Inhibited BASMC Proliferation Induced by Endothelin 1 or Hypotonic Medium via the MVA Pathway**

It has been established that SMC hyperplasia is one of the major causes of hypertension-induced vascular remodeling. So we examined the effect of simvastatin on BASMCs proliferation. Our results showed that simvastatin concentration-dependently inhibited BASMCs proliferation induced by 10 nmol/L of endothelin 1 (ET-1) and 25% hypotonic medium with estimated IC₅₀ values of 3.20±0.04 and 3.70±0.04 μmol/L (n=6 from 6 independent experiments per group), respectively (Figure S3).

To clarify whether simvastatin-elicited growth arrest was because of the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, we examined the effect of simvastatin on BASMC proliferation in the presence of MVA or its isoprenoid derivatives. MVA (100 μmol/L) or GGPP (10 μmol/L) treatment completely reversed the inhibitory effect of simvastatin on ET-1- or hypo-osmolarity–induced proliferation. FPP (10 μmol/L) also remarkably attenuated the inhibitory effect of simvastatin on cell proliferation (Figures 2A and 2B and S4). These results indicated that the growth arrest elicited by simvastatin was related to the inhibition of the MVA pathway.

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**Figure 2.** Effects of simvastatin (sim) and Rho/Rho-kinase inhibitors on ET-1 or hypo-osmolarity (Hypo) induced BASMC proliferation. Cell growth was analyzed by 5-bromodeoxyuridine incorporation. A and C, Effects of isoprenoids on simvastatin-induced inhibition of cell proliferation stimulated by ET-1 (A) or hypotonic medium (B), MVA (100 μmol/L), GGPP (10 μmol/L), or FPP (10 μmol/L) was added into the culture medium 1 hour before 10 μmol/L of simvastatin treatment. B and D, Effects of Rho/Rho-kinase inhibitors on ET-1 or hypo-osmolarity stimulated cell proliferation. RhoA inhibitor (0.25 μg/mL), clostridium botulinum C3 exoenzyme (C3), or 10 μmol/L of Rho kinase inhibitor Y-27632 was added 1 hour before cells exposure to ET-1 or hypotonic medium. *P<0.05 vs ET-1- or hypotonicity-treated group, #P<0.05 vs simvastatin-treated group (n=6 per group).
Figure 3. Effects of MVA, GGPP, or FPP on simvastatin-induced inhibition of hypotonicity-activated Cl\(^-\) movement and membrane current in BASMCs. A, i, Representative traces of hypotonicity-activated Cl\(^-\) efflux and current densities measured at -100 mV in each treatment group. MVA, GGPP, or FPP was added to the pipette solution. \(P<0.01\) vs control group; \#P<0.05, \##P<0.01 vs simvastatin only group (n=19 or 12, 10 \mu mol/L of MVA+10 \mu mol/L of sim (n=11), or 10 \mu mol/L of FPP+10 \mu mol/L of sim (n=7)). ii, Mean current densities measured at ±100 mV in each treatment group. MVA, GGPP, or FPP was added to the pipette solution. \(P<0.05\), **P<0.01 vs control group; \#P<0.05, \##P<0.01 vs simvastatin only group.

To further demonstrate the role of geranylgeranylated proteins, such as Rho, in cell proliferation, we investigated the effects of RhoA and Rho kinase inhibitors on proliferation of BASMCs. Pretreatment with RhoA inhibitor clostridium botulinum C3 exoenzyme (0.25 \mu g/mL) or Rho kinase inhibitor Y-27632 (10 \mu mol/L) attenuated the proliferation of BASMCs induced by ET-1 or 25\% hypotonic medium (Figures 2C and 2D and S5).

Simvastatin Inhibited the Activity of VRCC by Interfering With the MVA Pathway

Because VRCC has been documented to play an important role in the regulation of cell proliferation,\(^1\)\(^7\),\(^20\),\(^25\) we next examined the effect of simvastatin on the activity of VRCC in BASMCs. Pretreatment with simvastatin (2.5 to 10.0 \mu mol/L) indeed inhibited the hypo-osmolarity–activated Cl\(^-\) efflux and membrane Cl\(^-\) current. FPP also significantly attenuated the inhibitory effect of simvastatin on the activity of VRCC. However, MVA, GGPP, or FPP alone had no effects on the activity of the channel (Figure S8). These data indicated that GGPP-dependent isoprenylation of some signaling molecules, such as Rho GTPase, mediates the effects of simvastatin.

RhoA/Rho Kinase Inhibitors Reduced the Activity of VRCC

To further determine whether the RhoA/Rho kinase pathway regulates the activity of the VRCC, we examined the effects of C3 exoenzyme and Y-27632 on hypotonicity-activated Cl\(^-\) efflux and membrane Cl\(^-\) current. Modern approaches to simvastatin inhibited cell proliferation, as well as the activity of VRCC via the MVA pathway, we analyzed the
ClC-3 Overexpression Attenuated the Effect of Simvastatin on BASMC Proliferation

ClC-3 has been suggested to be the key component of VRCC in vascular SMCs. Our results in the present study showed that ET-1 or hypotonic medium treatment increased the expression ClC-3 in BASMCs, and simvastatin reduced these effects (Figure S9). To further investigate the functional role of ClC-3 in cell proliferation, we examined the effect of ClC-3 overexpression on proliferation by using ClC-3 cDNA transfection. Our results demonstrated that ClC-3 overexpression significantly increased cell proliferation in basal medium and enhanced proliferation induced by ET-1 or hypotonicity compared with that in the vector transfection group. Moreover, ClC-3 overexpression decreased the sup-

The inhibition ratio of simvastatin on ET-1 (A) or hypo-osmolarity (B) induced BASMC proliferation positively correlated with the inhibition ratio of simvastatin on hypotonicity-activated Cl⁻ efflux.

**Figure 4.** Effects of Rho/Rho-kinase inhibitors on hypotonicity-activated Cl⁻ movement and membrane current in BASMCs. A, i, Changes of [Cl⁻] induced by hypotonic solution in the absence (con) or presence of 0.25 μg/mL of Rho inhibitor C3 exoenzyme (C3). Cells were incubated with C3 for 4 hours before simvastatin treatment. *P<0.01 vs control group. n=30 cells from 6 independent experiments for each group. ii, I-V curves of hypotonicity-activated Cl⁻ current in the absence (con) or presence of 0.25 μg/mL of C3 in the pipette solution. iii, Mean current densities measured at ±100 mV under hypotonic solution in the absence (con) or presence of C3. *P<0.01 vs hypotonicity only group (n=6). B, i, changes of [Cl⁻] of Rho kinase inhibitor Y-27632. Cells were incubated with Y-27632 for 4 hours before simvastatin treatment. *P<0.01 vs hypotonicity only group (n=6). B, ii) iii)ii)

**Figure 5.** Correlation of the inhibitory effects of simvastatin on proliferation with those on hypotonicity-activated Cl⁻ efflux by using the inhibition ratios of simvastatin at various concentrations. Figure 5 demonstrated that the inhibition ratio of simvastatin on ET-1- or hypo-osmolarity–induced BASMC proliferation positively correlated with the inhibition ratio of simvastatin on hypotonicity-activated Cl⁻ efflux with correlation coefficients of 0.94 (n=30 cells from 6 independent experiments; P<0.001; Figure 5A) or 0.84 (n=30 cells from 6 independent experiments; P<0.001; Figure 5B), respectively, suggesting that VRCC may mediate the inhibitory effect of simvastatin on BASMC proliferation.
Simvastatin Inhibited the Increased Expression of CIC-3 in Rat Basilar Arteries During Hypertension

The immunohistochemical experiment showed that the fluorescent intensities of CIC-3 in basilar arteries were increased gradually with the development of cerebrovascular remodeling induced by hypertension. At 1, 4, 8, and 12 weeks in hypertensive rats, the values of the fluorescent intensity per micrometer squared were 114.7 ± 13.1, 134.4 ± 11.0, 170.9 ± 12.4, and 192.1 ± 17.3. The corresponding values in sham groups were 115.8 ± 10.8, 104.3 ± 9.1, 117.6 ± 8.7, and 111.8 ± 10.9. From 4 weeks after operation, CIC-3 expression in basilar arteries from hypertensive rats significantly increased compared with that in corresponding sham rats (n = 8 per group; P < 0.01). Simvastatin treatment normalized the increased expression of CIC-3 in hypertensive rats from 4 weeks after operation (Figure 7), and these results were further confirmed by Western blotting analysis (Figure S10).

Discussion

Vascular remodeling, an adaptative response to increased BP, is considered as one of the major contributors to cardiovascular diseases, such as stroke,1–3,5,26 Statins have been reported to be able to prevent the vascular remodeling of coronary artery, pulmonary artery, and carotid artery independent of their lipid-lowering effects.7,8,12,13,27 In this study, we found that the basilar artery exhibited a smaller lumen and external diameter and an increased media cross-sectional area and media:lumen ratio in 2-kidney 2-clip hypertensive rats from 4 weeks after operation. These structural changes were consistent with our previous results in the same hypertensive model and the other laboratory’s findings in spontaneously hypertensive rats.1,2,3,28 Simvastatin treatment prevented the alterations of the cerebrovascular structure from 4 weeks after operation. Moreover, simvastatin decreased BP at 8 and 12 weeks postoperatively by ∼30 and 50 mm Hg, respectively. Our findings that simvastatin reduced BP were in good agreement with several recent clinical trials and experimental animal studies that demonstrated that statins exhibited a modest BP-lowering effect in hypertensive subjects.29 Although the BPs in the simvastatin-treated group were not significantly different from those in the hypertension group at 1 and 4 weeks after operation, our results cannot exclude the possibility that the BP-lowering effect of simvastatin may contribute to its beneficial effects on cerebrovascular remodeling.
It is well established that abnormal SMC proliferation contributes to hypertension-induced vascular remodeling. Statins have been demonstrated to inhibit SMC proliferation in vitro and in vivo independent of lipid-lowering effects and, thus, to ameliorate vascular proliferative disease. The pleiotropic effects of statins on proliferation and migration of SMC are considered to be derived from inhibition of isoprenoid intermediates, which serve as the lipid attachments for the posttranslational modification of a variety of proteins, such as those in the Rho and Rac GTPase family. In the present study, simvastatin inhibited the cultured rat BASMC proliferation induced by hypotonic medium and ET-1. MVA and GGPP preincubation abolished the inhibitory effect of simvastatin on cell proliferation. These data demonstrated that simvastatin inhibited BASMC proliferation via modifying Rho geranylgeranylation. Although several molecules, such as p21 and extracellular regulated kinase, have been reported to mediate the functional roles of Rho signaling in the regulation of cell proliferation, the downstream effectors of active Rho remain elusive.

During the early phase of cell proliferation, cells undergo swelling because of water influx, which accompanies the uptake of nutrients necessary for cell metabolism. An increase in cell volume will initiate the regulatory volume decrease process through activation of ion (K and Cl) channels and transporters, which activates K and Cl efflux to normalize the cell size. VRCC contributes to the regulatory volume decrease process; therefore, this channel has been suggested to play a critical role in the regulation of cell proliferation. Our previous studies have demonstrated that pharmacological inhibition of VRCC or knockdown of CIC-3 reduced rat aortic SMC proliferation induced by ET-1. In addition, our recent work in rat BASMCs demonstrated that CIC-3 silence inhibited cell proliferation and cell cycle progression. Our results in this study showed that hypotonicity–induced cell swelling stimulated rat BASMC proliferation, further supporting the functional role of VRCC in the regulation of cell growth.

The pleiotropic effects of statins on proliferation and migration of SMC have been suggested to play a critical role in the regulation of cell proliferation. These data demonstrated that ET-1 and hypotonic medium increased cell proliferation, as well as ClC-3 expression, and simvastatin treatment inhibited the upregulation of CIC-3 caused by ET-1 and hypo-osmolarity. In addition, the expression of CIC-3 in rat BASMCs because the Rho and Rho kinase inhibitors blocked hypo-osmolarity–induced chloride efflux and transmembrane chloride current. Because statins modify the activity of Rho GTPase, we next tested the effect of simvastatin on the activation of VRCC. We found that simvastatin inhibited hypotonicity-activated chloride movement and volume-regulated chloride current in a concentration-dependent manner. Similar results were also observed after lovastatin treatment (data not shown). The inhibitory effect of simvastatin on activation of VRCC was abolished by MVA and GGPP pretreatment, suggesting that simvastatin inhibited VRCC via modifying Rho activity.

Vascular SMC volume increase has been suggested to be the primary change responsible for the hypertrophy of mesenteric arterial media in spontaneously hypertensive rats. In BASMCs, we found that the cell volume was increased during the development of hypertension in hypertensive rats. In addition, the activity of VRCC is also increased during hypertension, and its increment parallels the severity of the vascular remodeling, suggesting that VRCC may be associated with the abnormal vascular SMC proliferation and vascular remodeling during hypertension. Our present work demonstrated that ET-1 and hypotonic medium increased cell proliferation, as well as CIC-3 expression, and simvastatin treatment inhibited the upregulation of CIC-3 caused by ET-1 and hypo-osmolarity. In addition, the expression of CIC-3 in basal arteries was increased gradually with the development of cerebrovascular remodeling induced by hypertension. Simvastatin treatment normalized the structural alterations, as well as CIC-3 expression, in the basilar artery of hypertensive rats. Moreover, CIC-3 overexpression in BASMCs decreased the inhibitory effect of simvastatin on cell proliferation. These findings indicated that inhibition of VRCC underlies the beneficial effects of simvastatin on vascular remodeling during hypertension.

**Perspectives**

VRCC has been suggested to play an important role in the regulation of cell volume, proliferation, and apoptosis. The
present study provides new mechanistic insights that inhibition of VRCC contributes to the beneficial effects of simvastatin on cerebrovascular remodeling. The data suggest that VRCC is a potential target for the prevention of cerebrovascu-
lar remodeling and stroke. Future studies are needed to investigate the functional role, as well as the mechanisms, of VRCC in the development of vascular remodeling during hypertension in ClCn-3 transgenic and knockout mice.

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Disclosures
None.

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Simvastatin ameliorates rat cerebrovascular remodeling during hypertension via inhibition of volume-regulated chloride channel

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Running title: Simvastatin and volume-regulated chloride channel

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ONLINE SUPPLEMENT

Materials and Methods

Cell culture medium, fetal calf serum and BrdU Assay Kit were provided by Invitrogen. Mevalonate, geranylgeranylprenylphosphate (GGPP), farnesylphosphorylphosphate (FPP), Clostridium botulinum C3 exoenzyme, Y-27632 ((+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)), α-actin monoclonal antibody was purchased from Sigma. 6-Methoxy-N-ethylquinolinium iodide (MEQ) were from Molecular Probes. ClC-3 rabbit polyclonal antibody was provided by Alomone Labs (Jerusalem, Israel). gpClC-3/pcDNA3.1 was kindly provided by Dr. J.R. Hume (University of Nevada School of Medicine, Reno, Nevada, USA).

Simvastatin was kindly provided by Merck Sharp & Dohme (Hangzhou, China) and was prepared for in vitro use as described previously. Briefly, the inactive lactone was converted into the active form by dissolving 20 mg lactone in 0.5 ml 100% ethanol, after adding 0.75 µl 0.1 mol/L NaOH, the solution was heated at 50°C for 2 h. The pH of the solution was adjusted to 7.2 with 0.1 mol/L HCl and then simvastatin was diluted to 5 mmol/L stock solution with PBS.

Animals were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All experimental procedures were performed in accordance with the policies of the Sun Yat-Sen University Animal Care and Use Committee and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China.

Animal model and blood pressure measurement

2-kidney, 2 clip (2k2c) renovascular stroke-prone hypertensive rats were operated as previously described. Briefly, healthy male Sprague-Dawley rats (90-120g) were anaesthetized by injection of 10% chloral hydrate (3 mg/kg, 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. Systolic blood pressure (SBP) was measured in conscious rats by tail-cuff plethysmography (powerlab 4/30, ADInstruments, Australia). Simvastatin (Merk Sharp & Dohme Ltd. Northumberland, UK) was dissolved in water and orally administered by gavage at a daily dose of 10 mg/kg after operation. All rats were maintained in pathogen-free facilities with a 12-hour light/dark cycle.

Immunohistochemistry

Rats were anesthetized with 10% chloralhydrat and were perfused intracardiacally with 0.1 mol/L phosphate buffer containing heparin (100 U/kg) and nitroglycerol (0.3 µg/kg), followed by 4°C fixative solution containing 4% freshly depolymerized paraformaldehyde in 0.1 mol/L phosphate buffer for 15
minutes, the pressure was controlled ≈100mmHg. The rat brain was carefully removed and sections (8 µm) were prepared from freshly frozen rat basilar arteries as previously described³,⁴. 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 30min at room temperature, and then blocked with 5% bovine serum albumin in PBS for 30min. Then the sections were exposed to α-actin monoclonal antibody (Sigma; dilution 1:400) or ClC-3 rabbit polyclonal antibody (Alomone Labs; dilution 1:100) at 4°C overnight and then were treated with goat FITC-conjugated secondary antibody (Cell Signaling Technology; dilution 1:400) or Alexa Fluor® 488 goat anti-rabbit IgG (Cell Signaling Technology; dilution 1:400) at room temperature for 30min. The quantification of α-SM-actin staining was analyzed 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.

**Electron microscopy**

Rats were anesthetized with 10% chloralhydrat and were perfused intracardiacly with 0.1 mol/L phosphate buffer containing heparin (100 U/kg) and nitroglycerol (0.3 µg/kg), followed by 4°C fixative solution containing 4% paraformaldehyde, 0.25% glutaral, 15% saturated trinitrophenol in 0.1 mol/L phosphate buffer for 15 minutes, the pressure was controlled ≈100mmHg. The brain tissue containing the basilar artery at midpoint were removed and were cut into blocks of 1mm×1mm×3mm, and then immersed into fixative solution overnight at 4°C. The tissue blocks were postfixed in 2% osmic acid for 2h, dehydrated via graded alcohols and embedded in Epon 812. Ultrathin sections with thickness of 80~100nm were prepared and stained with uranyl acetate and lead citrate before they were viewed with a transmission H-600 electron microscope (Hitachi, Japan).

**Cell culture**

Rat basilar smooth muscle cells (BASMCs) were isolated and cultured from rat basilar arteries using explant method as previously described⁵-⁸. Briefly, male Sprague-Dawley rats (150~180g) were anaesthetized with ether and decapitated. The basilar arteries were harvested immediately and immersed in Kreb's solution containing (mmol/L): NaCl 137, KCl 5.4, CaCl₂ 2.0, MgCl₂ 1.1, NaH₂PO₄ 0.4, Glucose 5.6, NaHCO₃ 11.9, 10⁵ U/L penicillin and 100 mg/L streptomycin. After fat and connective tissue was cleaned, the basilar arteries were cut into small pieces about 0.5 mm long and the vessel segments were placed on the surface of the culture dish. The dish was then incubated in Dulbecco's Modified Essential Medium (DMEM)/F-12 supplemented with 20% fetal calf serum (FCS) at 37°C, 5% CO₂. 3 days later, the floating tissues were discarded and fresh DMEM/F-12 containing 20% FCS was added. The cells will migrate from the attached vascular specimens about 7-10 days. After reaching 70% confluence, cells were then subcultured with 0.25% trypsin with
0.02% EDTA. Passage 8 to 12 of BASMCs at 70% to 90% confluence were growth-arrested by incubation in DMEM/F12 with 0.5% FCS for 24 hours before experiments. BASMCs were identified by positive immunocytochemical staining with a monoclonal antibody against smooth muscle α-actin and by the characteristic “hill and valley” growth pattern.

**Proliferation assays**

Cell proliferation was assessed by cell counts and 5-bromo-2'-deoxyuridine (BrdU) incorporation as we reported previously. For the cell counts, BASMCs were trypsinized and plated into 6-well culture plates at a density of 5×10^5 cells/well in DMEM/F-12 supplemented with 10% FCS. After 24 hours, cells were rendered quiescent in 0.5% FCS for 24 hours before addition of fresh growth medium containing the appropriate supplements. Cell number was determined in triplicate using a hemocytometer.

The DNA synthesis was assessed by BrdU incorporation. After starvation for 24 hours, cells were treated with the same supplements used in the cell count experiment before adding 10 mmol/L BrdU to the medium. 18 hours later, cells were fixed and treated with anti-BrdU primary antibody for 1 h at room temperature. After incubation with horseradish peroxidase-conjugated goat anti-IgG for 30 min, 100 mmol/L 3,3′,5,5′- tetramethylbensidine was then added as the substrate for horseradish peroxidase. The incorporation was measured at 450 and 540nm on an Elisa microplate reader (BIO-TEK synergy HT, American).

The inhibition ratios of simvastatin (sim, 2.5-10 μmol/L) on ET-1 or 25% hypotonic medium (hypo) induced proliferation were calculated as follows:

\[
\frac{\text{OD}_{\text{ET-1}} - \text{OD}_{\text{ET-1+sim}}}{\text{OD}_{\text{ET-1}} - \text{OD}_{\text{control}}} \times 100\% \quad \text{or} \\
\frac{\text{OD}_{\text{hypo}} - \text{OD}_{\text{hypo+sim}}}{\text{OD}_{\text{hypo}} - \text{OD}_{\text{control}}} \times 100\%
\]

**gpClC-3 cDNA transfection**

gpClC-3/cDNA3.1 plasmid was transfected as previously described. Briefly, BASMCs were plated on 24-well plate at a density of 1-1.5×10^5/ml. 24 hours later, gpClC-3/cDNA3.1 plasmid were tansfected into the cells with LipofectAMINE reagent (Invitrogen, Life Technologies, Inc.) in OPTI-MEM reduced serum medium (GIBICO) according to the manufacturer’s instructions. 6h later, cells were rinsed with PBS and switched to 0.5% serum-containing medium for cell proliferation assays.

**Measurement of [Cl]_i**

[Cl]_i was measured with 6-Methoxy-N-ethylquinolinium iodide (MEQ) as we described previously. Briefly, MEQ was first reduced to a cell-permeable form, 6-methoxy-N-ethyl-1,2-dihydroquinoline (diH-MEQ). Then Cells were incubated with 100-150 μmol/L diH-MEQ in a Ringer’s buffer solution containing (mmol/L) :119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 MgSO_4, 2.5 CaCl_2, 26 NaHCO_3 and 11 Glucose, pH 7.4 at room temperature in the dark for 30min. [Cl]_i was monitored by MetaFluor Imaging software (Universal Imaging Systems, Chester, PA) with 350 nm excitation wavelength and 435 nm emission wavelength. Relationship between fluorescence intensity of MEQ
and chloride concentration is given by the Stern–Volmer equation: \((F_0/F) - 1 = K_{SV} [Q]\). Where \(F_0\) is the fluorescence intensity without halide or other quenching ions; \(F\) is the fluorescence intensity in the presence of quencher; \([Q]\) is the concentration of quencher; and \(K_{SV}\) is the Stern–Volmer constant.

Solutions: The osmolarities of the solutions were measured by a freezing point depression osmometer (OSMOMAT030, Germany). The isotonic bath solution (300 mosmol/kg\(\cdot\)H\(_2\)O) contained (mmol/L): 111 NaCl, 2.5 KCl, 0.5 MgCl\(_2\), 10 HEPES, 5 glucose, and 70 sucrose, pH 7.4 with NaOH. The 230 mosmol/kg\(\cdot\)H\(_2\)O hypotonic solution was prepared by omitting the sucrose from the isotonic solution.

The inhibition ratios of simvastatin (sim, 2.5-10 \(\mu\)mol/L) on hypotonicity (hypo) activated Cl\(^-\) efflux were calculated as follow:

\[
\frac{[(\text{Cl}^-)_{\text{iso}} - (\text{Cl}^-)_{\text{hypo}}]_{\text{con}} - [(\text{Cl}^-)_{\text{iso}} - (\text{Cl}^-)_{\text{hypo}}]_{\text{sim}}}{[(\text{Cl}^-)_{\text{iso}} - (\text{Cl}^-)_{\text{hypo}}]_{\text{con}}} \times 100\%
\]

**Electrophysiological experiments**

Membrane whole-cell Cl\(^-\) currents were recorded with an Axopatch 200B Amplifier (Axon Instrument, Foster City, CA) as previously described\(^1\). The isotonic extracellular solution contained (mmol/L): 107 N-methyl-D-glucamine chloride (NMDG-Cl), 1.5 MgCl\(_2\), 2.5 MnCl\(_2\), 0.5 CdCl\(_2\), 0.05 GdCl\(_3\), 10 glucose, 10 HEPES, and 70 D-mannitol, pH 7.4 with NMDG. The osmolarity was 300 mosmol/kg\(\cdot\)H\(_2\)O. The hypotonic solution was made by omitting D-mannitol from the isotonic solution with an osmolarity of 230 mosmol/kg\(\cdot\)H\(_2\)O. Internal pipette solution (300 mosmol/kg\(\cdot\)H\(_2\)O) contained (mmol/L): 95 CsCl, 20 TEACl, 5 ATP-Mg, 5 EGTA, 5 HEPES, and 80 D-mannitol, pH 7.2 with CsOH. Recordings were started 5 min after the establishment of the whole-cell configuration to allow the equilibration of the pipette solution with cell interior. The currents were elicited with voltage steps from -100mV to +120mV in +20mV increment for 400ms with an interval of 5s from a holding potential of -40mV. Currents were sampled at 5 kHz using pCLAMP8.0 software (Axon Instruments) and filtered at 2 kHz. To minimize the changes of liquid junction potentials, a 3 mM KCl-agar salt bridge between the bath and the Ag-AgCl reference electrode was used. All experiments were performed at room temperature (25°C).

**Western blot analysis**

Total proteins were prepared from the cultured BASMCs or pooled basilar arteries from sham group, hypertensive rats with or without simvastatin treatment (4 basilar arteries from each group were used as 1 sample), and western blotting was performed as previously described\(^10,\ 11\). Briefly, the protein content was quantified with Coomassie Brilliant Blue. Aliquots containing 30 \(\mu\)g of proteins from each group were subjected to SDS-PAGE and were then transferred to nitrocellulose membranes (Schleicher & Schuell). After incubation with the blocking solution at room temperature for 1 hour, the membranes were incubated with a rabbit antibody against CIC-3 diluted 1:400 for 1 hour at room temperature, and then incubated with a horseradish
peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology; dilution 1:1000) for 1 hour at room temperature. Final detection was carried out with LumiGLO chemiluminescent reagent (New England Biolabs) as described by the manufacturer. The densities of target bands was accurately determined by the computer-aided 1-D gel analysis system.

**Statistical Analysis**

All data were expressed as mean± SEM. Statistical analyses were performed using Student’s t test or ANOVA. Values of P < 0.05 were considered significant.

**References**


Figure S1. Simvastatin ameliorated the morphological changes of rat cerebral basilar artery during hypertension. Vascular media smooth muscle was specific stained as brown with α-smooth muscle actin antibody. Representative pictures were taken from the rat basilar arteries of control (Con), hypertensive rats without (Htn) or with simvastatin (Sim) treatment at 1w, 4w, 8w and 12w after operation, respectively (×200). At each time point, 6 rats were studied in
each group.

Figure S2. Electron microscopy analysis of the effects of simvastatin on rat cerebral basilar artery during hypertension. Representative pictures were taken from control (Con), hypertensive rats without (Htn) or with simvastatin (Sim) treatment at 1w, 4w, 8w and 12w after operation, respectively. Black bar represents 2 µm. Vacuolization (◇), fibrosis (△), and apoptosis of smooth muscle cells ((newUser:latex){\varphi}) in the basilar artery of hypertensive rats were ameliorated by simvastatin treatment. At each time point, 6 rats were studied in each group.
Figure S3. Effect of simvastatin (sim) on endothelin-1 (ET-1) or hypoosmolarity (Hypo) induced proliferation of BASMCs. Cell proliferation was assayed by BrdU incorporation (A and C) and by cell counts (B and D). Simvastatin (0.6-10 μmol/L) inhibited BASMC proliferation induced by 10 nmol/L ET-1 or 25% hypotonic medium (Hypo) in a concentration-dependent manner. Simvastatin was added to the cell culture medium 1 hour before exposure to ET-1 or hypotonic medium.
Figure S4. Effects of isoprenoids on simvastatin-induced inhibition of cell proliferation stimulated by ET-1 (A) or hypotonic medium (B). Cell growth was analyzed by means of cell counts. Mevalonate (MVA, 100 μmol/L), geranylgeranylpyrophosphate (GGPP, 10 μmol/L), or farnesylpyrophosphate (FPP, 10 μmol/L) was added to the culture medium 1 hour before 10 μmol/L simvastatin treatment. * p < 0.05 vs ET-1- or hypotonicity-treated group, # p < 0.05 vs simvastatin-treated group (n=6 per group).
Figure S5. Effects of Rho/Rho-kinase inhibitors on ET-1 (A) or hypotonic medium (Hypo) (B) induced BASMC proliferation. Cell growth was analyzed by cell counts. 0.25 μg/ml RhoA inhibitor, Clostridium botulinum C3 exoenzyme (C3), or 10 μmol/L Rho kinase inhibitor, Y-27632, was added 1 hour before cells exposure to ET-1 or hypotonic medium. * p < 0.05 vs simvastatin-treated group (n=6 per group).
Figure S6. Effects of simvastatin (sim) on hypotonicity-activated Cl\(^{-}\) movement in BASMCs. A. representative traces of hypotonicity-activated Cl\(^{-}\) efflux in the absence (con) or presence of simvastatin (2.5-10 μmol/L). B. simvastatin concentration-dependently inhibited hypotonicity-activated Cl\(^{-}\) efflux but did not affect intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]\(_{i}\)) under isotonic condition. The cells were preincubated with simvastatin in isotonic solution for 5 min before exposure to hypotonic solution. *p < 0.05 vs control group (n=100 cells from 6 independent experiments per group).
Figure S7. Effects of simvastatin (sim) on hypotonicity-activated membrane current in BASMCs. A. representative traces of the hypotonicity-activated Cl⁻ current in the absence (con) or presence of simvastatin (2.5-10 μmol/L). B. I-V curves of hypotonicity-activated Cl⁻ current in the absence (con) or presence of simvastatin (2.5-10 μmol/L). The cells were preincubated with simvastatin in isotonic solution for 5 min before exposure to hypotonic solution. V. mean current densities measured at ±100mV under hypotonic solution from BASMCs in the absence (con) or presence of simvastatin. The current density was represented as pA/pF. * p < 0.05, **p<0.01 vs control group (n=10 to 15 per group).
Figure S8. MVA, GGPP or FPP alone had no effects on hypotonicity-activated Cl⁻ movement (A) and membrane current (B) in BASMCs. MVA (100 μmol/L), GGPP (10 μmol/L) or FPP (10 μmol/L) was added to the culture medium 1 hour before measuring hypotonicity-activated Cl⁻ movement and membrane current.
**Figure S9**. Effects of simvastatin on ET-1 or hypotonic medium induced upregulation of CIC-3 in BASMCs. Cells were treated with 10 nmol/L ET-1 or 25% hypotonic medium (Hypo) with or without 10 μmol/L simvastatin and protein was harvested 48 hours later. Western blot of cell lysates demonstrated that ET-1 (A) and hypotonic medium (B) both increased CIC-3 expression. Simvastatin (sim) reversed the effects of ET-1 and hypotonic medium on CIC-3 expression. * p < 0.01 vs control, # p < 0.01 vs ET-1- or hypotonicity-treated group (n=4 per group).
Fig S10

**Figure S10.** Simvastatin normalized the increased expression of CIC-3 in rat basilar artery during hypertension. Basilar arteries were collected from control, hypertensive rats with or without simvastatin treatment. Tissue protein was prepared and subjected to SDS-PAGE. A. Western blot analysis showed that, from 4 week after operation, the CIC-3 protein expression in basilar artery from hypertensive rat was increased compared with the corresponding sham rat (*p<0.01 vs corresponding sham group, n=4 from 12 rats per group). B. Simvastatin attenuated the increased expression of CIC-3 in hypertensive rats at 12 weeks after operation (*p<0.01 vs sham group, #p<0.01 vs hypertension group, n=4 from 12 rats per group).