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

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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 Rac, 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 IC50 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-
nel,\textsuperscript{15} and nonselective cation channel,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{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.\textsuperscript{20} These cells were used to study the cell proliferation and to measure intracellular Cl\textsuperscript{−} concentration ([Cl\textsuperscript{−}]\textsubscript{i}) and transmembrane Cl\textsuperscript{−} currents, as described previously.\textsuperscript{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,\textsuperscript{21} the BP in the hypertensive group was significantly higher than in the sham group. The effects of simvastatin on BP are shown in Table 1. Simvastatin significantly reduced BP in the hypertension + simvastatin group compared to the hypertension group. The results suggest that simvastatin may have a beneficial effect on the cerebrovascular remodeling in the hypertension model.

![Figure 1. Structure parameters of rat cerebral basilar arteries from sham, hypertension, and hypertension treated with simvastatin (Sim) groups. A. CSA indicates cross-sectional area. B. LD indicates lumen diameter. C. WD indicates wall diameter. D. W/L indicates wall: lumen ratio. *$P<0.05$, **$P<0.01$ vs corresponding sham groups; #$P<0.05$, ##$P<0.01$ vs corresponding hypertension group. Values are mean±SEM in sections from 6 rats of each group.](image)

**Table. Effects of Simvastatin on BP**

| Group   | N  | 1 wk | 4 wk | 8 wk | 12 wk |
|---------|----|------|------|------|-------|
| Sham    | 10 | 99.0±3.1 | 102.5±2.8 | 105.5±2.7 | 106.5±3.0 |
| Htn     | 10 | 107.0±3.7 | 146.0±5.0* | 172.5±4.6* | 200.5±4.8* |
| Htn+Sim | 10 | 107.5±2.9 | 132.5±5.1* | 140.5±3.7† | 147.0±4.2‡ |

Data are mean±SEM in millimeters of mercury. Htn indicates hypertension; Sim, simvastatin.

* $P<0.01$ vs corresponding sham group.
† $P<0.01$ vs corresponding hypertension 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 corresponding control groups. The wall diameter decreased and the wall:lumen area ratio and the mean arterial pressure decreased 4 weeks postoperatively compared with those in the hypertension group after 8 weeks postoperatively (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 IC_{50} 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.
MVA inhibited the hypo-osmolarity–activated Cl\(^{-}\) current in BASMCs. A, i, Representative traces of hypotonicity-activated Cl\(^{-}\) current densities measured at **[Cl\(-\)]\(_{i}\)** in each treatment group. MVA, GGPP, or FPP was added to the isotonic extracellular solution 5 minutes before simvastatin (sim), 100 \(\mu\)mol/L of MVA, 10 \(\mu\)mol/L of GGPP, or 10 \(\mu\)mol/L of FPP was added to the culture medium 1 hour before simvastatin treatment. *P<0.01 vs control group; #P<0.05, ##P<0.01 vs simvastatin only group (n=30 cells from 6 independent experiments per group). B, i, I-V curves of hypotonicity-activated Cl\(^{-}\) current evoked by hypotonic medium when applying the pipette solution (Figure S8). These data indicated that GGPP- and FPP-dependent isoprenylation of some signaling molecules, such as Rho GT-Pase, mediates the effects of simvastatin.

**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, \(^{17,20,25}\) we next examined 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).

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) remarkably reduced membrane 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 GT-Pase, 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. Pretreatment with C3 exoenzyme (0.25 \(\mu\)g/mL) or Y-27632 (10 \(\mu\)mol/L) for 4 hours significantly inhibited Cl\(^{-}\) efflux stimulated by hypotonic solution. In addition, C3 exoenzyme (0.25 \(\mu\)g/mL) or Y-27632 (10 \(\mu\)mol/L) remarkably reduced membrane Cl\(^{-}\) current evoked by hypotonic medium when applying the pipette solution (Figure 4A and 4B).

**Simvastatin-Elicited Inhibition of Proliferation Positively Correlated With Its Suppressive Effect on Cl\(^{-}\) Efflux**

Because simvastatin inhibited cell proliferation, as well as the activity of VRCC via the MVA pathway, we analyzed the
ClC-3 overexpression decreased the sup-

omosmolarity compared with that in the vector transfection and enhanced proliferation induced by ET-1 or hypo-

tonic medium treatment. Our results demonstrated that ClC-3 overexpres-
sion ClC-3 in cell proliferation, we examined the effect of ef-
sions (Figure S9). To further investigate the functional role of ClC-3 in BASMCs, and simvastatin reduced these ef-
sions.

correlation of the inhibitory effects of simvastatin on prolif-
eration with those on hypotonicity-activated Cl\(^-\) efflux by using the inhibition ratios of simvastatin at various concen-
trations. Figure 5 demonstrated that the inhibition ratio of simvastatin on ET-1- or hypo-osmolarity–induced BASMC prolif-
eration positively correlated with the inhibition ratio of simvastatin on hypotonicity-activated Cl\(^-\) efflux with corre-
lation coefficients of 0.94 (n=30 cells from 6 independent experiments; \(P<0.001\); Figure 5A) or 0.84 (n=30 cells from 6 inde-
pendent experiments; \(P<0.001\); Figure 5B), respec-
tively, suggesting that VRCC may mediate the inhibitory effect of simvastatin on BASMC proliferation.

**CIC-3 Overexpression Attenuated the Effect of Simvastatin on BASMC Proliferation**

CIC-3 has been suggested to be the key component of VRCC in vascular SMCs.\(^{22}\) Our results in the present study showed that ET-1 or hypotonic medium treatment increased the expression CIC-3 in BASMCs, and simvastatin reduced these effects (Figure S9). To further investigate the functional role of CIC-3 in cell proliferation, we examined the effect of CIC-3 overexpression on proliferation by using CIC-3 cDNA transfection. Our results demonstrated that CIC-3 overexpres-
sion significantly increased cell proliferation in basal medium and enhanced proliferation induced by ET-1 or hypo-
osmolarity compared with that in the vector transfection group. Moreover, CIC-3 overexpression decreased the sup-

![Figure 4](image-url)  
**Figure 4.** Effects of Rho/Rho-kinase inhibitors on hypotonicity-activated Cl\(^-\) movement and membrane current in BASMCs. A, i, Changes of [Cl\(^-\)]\(_i\) induced by hypotonic solution in the absence (con) or presence of 0.25 \(\mu\)g/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 \(\mu\)g/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\(^-\)]\(_i\) induced by hypotonic solution in the absence (con) or presence of 0.25 \(\mu\)g/mL of Rho kinase inhibitor Y-27632. Cells were incubated with Y-27632 for 4 hours before simvastatin treatment. \(^*P<0.01\) vs control group (n=30 cells from 6 independent experiments per group). ii, I-V curves of hypotonicity-activated Cl\(^-\) current in the absence (con) or presence of 10 \(\mu\)mol/L of Rho kinase inhibitor Y-27632. Cells were incubated with Y-27632 for 4 hours before simvastatin treatment. \(^*P<0.01\) vs control group (n=30 cells from 6 independent experiments per group). ii, I-V curves of hypotonicity-activated Cl\(^-\) current in the absence (con) or presence of 10 \(\mu\)mol/L of Y-27632 in the pipette solution. iii, Mean current densities measured at \(+100\) mV under iso-
tonic and hypotonic solution in the absence (con) or presence of Y-27632. \(^*P<0.01\) vs hypotonicity only group (n=6).

![Figure 5](image-url)  
**Figure 5.** 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.
and hypo-osmolarity-induced proliferation from 3.40 respectively (P<0.05, **P<0.01 vs vector transfection group (n=6).

**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.7,8,10,12,13,27 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 SMCs 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 activity.

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 Rho-Rho kinase pathway has been documented to be involved in the regulation of volume-regulated chloride current in NIH3T3 mouse fibroblasts and pulmonary artery endothelial cells. Our data in this study showed that the Rho-Rho kinase pathway is necessary for the activation of VRCC in rat BASMCs because the Rho and Rho kinase inhibitors blocked hypotonicity–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 basilar 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.
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 cerebrovascular 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 CICn-3 transgenic and knockout mice.

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Disclosures
None.

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