Statins Promote the Growth of Experimentally Induced Cerebral Aneurysms in Estrogen-Deficient Rats

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Background and Purpose—The pathogenesis of cerebral aneurysms is linked to inflammation, degradation of the extracellular matrix, and vascular wall apoptosis. Statins exert pleiotropic effects on the vasculature, independent of their cholesterol-lowering properties. To explore the detailed pathogenesis of cerebral aneurysms, we examined their progression in a rat model and studied whether statins prevent their initiation and growth.

Methods—Cerebral aneurysms were induced in female rats subjected to hypertension, increased hemodynamic stress, and estrogen deficiency. The development of aneurysm was assessed morphologically on corrosion casts. The effects of pravastatin (5, 25, or 50 mg/kg per day) and of simvastatin (5 mg/kg per day) on their aneurysms were studied. Human brain endothelial cells were also used to determine the effects of pravastatin.

Results—Pravastatin (5 mg/kg per day) reduced endothelial damage and inhibited aneurysm formation; there was an association with increased endothelial nitric oxide synthase (eNOS) levels and a decrease in human brain endothelial cell adhesion molecules. Unexpectedly, 25 mg/kg per day and 50 mg/kg per day pravastatin and 5 mg/kg per day simvastatin promoted aneurysmal growth, and high-dose pravastatin induced aneurysmal rupture. The deleterious effects exerted by these statins were associated with an increase in apoptotic caspase-3 levels and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells, suggesting that statins exert bidirectional effects.

Conclusions—Our results provide the first evidence that cerebral aneurysm growth is partly associated with apoptosis and issue a warning that statins exert bidirectional effects on cerebral aneurysms. Additional intensive research is necessary to understand better their mechanisms and to identify patients in whom the administration of statins may elicit deleterious effects. (Stroke. 2011;42:2286-2293.)

Key Words: cerebral aneurysm ■ statin ■ apoptosis ■ rat

Subarachnoid hemorrhage (SAH) caused by the rupture of cerebral aneurysms carries a high mortality rate, and endovascular or intracranial surgery (coiling or clipping) is performed to prevent their rupture. To date, no drugs to address cerebral aneurysms have been approved because the detailed pathogenesis with respect to their initiation, progression, and rupture remains to be elucidated.

Based on epidemiological data showing a high incidence of cerebral aneurysms in postmenopausal women, we developed an experimental model in female rats subjected to increased hemodynamic stress, hypertension, and estrogen deficiency by oophorectomy (OVX). Using this model, we found that endothelial injury is an initial event in the pathogenesis of cerebral aneurysms, and that infiltration of leukocytes from interendothelial gaps, where tight junction proteins are reduced, is associated with their progression. We also demonstrated that a reduction in endothelial nitric oxide synthase (eNOS) messenger ribonucleic acid (mRNA), an elevation in nicotinamide adenine dinucleotide phosphate oxidase and angiotensin II levels, and inflammation mediated by matrix metalloproteinase-9 derived from macrophages in the vascular wall, are associated with the formation of cerebral aneurysms. In contrast, various types of agents including 17β-estradiol, an angiotensin II type 1 receptor blocker, a phosphodiesterase 4 inhibitor, and the mineralocorticoid receptor antagonist eplerenone, effectively prevented the formation of cerebral aneurysms via their antioxidative and anti-inflammatory effects.

As all types of statins exert beneficial antioxidant and anti-inflammatory effects, they may protect against endothelial dysfunction and the development and progression of atherosclerosis. The efficacy of some statins in experimental cerebral aneurysm models has been demonstrated. However, the effects of statins on vascular smooth muscle cells...
(VSMC) and endothelial cells remain controversial and depend on their hydrophilic or lipophilic nature, doses administered, and the length of treatment.

Here we provide new evidence that hydrophilic pravastatin exerts bidirectional effects on cerebral aneurysms in hypertensive, estrogen-deficient female rats; at low doses, it inhibited endothelial damage, thereby preventing aneurysmal progression. However, unexpectedly, at high doses, pravastatin as well as lipophilic simvastatin, promoted development and growth of cerebral aneurysms. We demonstrate that deleterious effects of statins are at least partly associated with the induction of apoptosis in the aneurysmal wall.

Materials and Methods
For a detailed description, please see the online data supplement at http://stroke.ahajournals.org.

Experiment 1: Time Course of Aneurysmal Changes at the Left ACA-OA Bifurcation
Rats exposed to renal ligation, hemodynamic stress, and estrogen deficiency were sacrificed 3, 6, 12, and 24 weeks later for morphological assessments.

Experiment 2: Effects of High-Dose Pravastatin on Rat Cerebral Aneurysms
To examine whether pravastatin prevents the formation of aneurysms by improving endothelial dysfunction, we began treating rats with the drug 2 weeks before OVX. They were randomly divided into a vehicle control (VC) group (n=7) that received 5% Arabic gum solution and 25 and 50 mg/kg/day pravastatin groups (P-25 and P-50 group, n=12 each).

Experiment 3: Comparison of the Effect of Low-Dose Pravastatin and Simvastatin on Aneurysm Formation
Rats were randomly assigned to 3 groups (supplemental Figure S1) and received 5% Arabic gum solution (VC group, n=8), low-dose pravastatin (5 mg/kg/day, P-5, n=12), or simvastatin in 5% Arabic gum solution (5 mg/kg/day, Sim-5 group, n=13) for 14 weeks.

Experiment 4
We determined the formation and progression of cerebral aneurysms in rats treated with 50 mg/kg pravastatin alone (P-50 group, n=7) or with pravastatin plus CoQ10 (15 mg/kg/day, CoQ10 group, n=12).

Preparation and Study of Corrosion Casts
Vascular corrosion casts were prepared as previously described.2,3,5–7 The left anterior cerebral artery-olfactory artery (ACA-OA) bifurcation was inspected at 3kV under a scanning electron microscope (SEM; VE8800, Keyence, Osaka, Japan) and changes were recorded as normal (stage 0), endothelial damage (stage 1), moderate protrusion (stage 2), and sacular aneurysm (stage 3). Morphological changes at the bifurcation were evaluated by 3 blinded observers and staged as described elsewhere.2,3,5–7

Immunohistochemistry and TUNEL Staining
Sham-operated (n=6), VC (n=6), P-5 (n=7), P-50 (n=7), and Sim-5 rats (n=7) at 12 weeks after OVX (at 23 weeks of age) were used for immunohistochemical study. Primary polyclonal antibodies against cleaved caspase-3 and monoclonal antibody against anti-smooth muscle β-actin (Laboratory Vision, CA) were used. To assess apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed.

Quantitative Real-Time PCR (qRT-PCR)
Samples from sham-operated (n=6), VC (n=6), P-5 (n=7), P-50 (n=7), and Sim-5 rats (n=7), were subjected to quantitative real-time PCR (qRT-PCR) using total RNA from the left ACA-OA as previously described.2,3,5–7 qRT-PCR of each sample was on a LightCycler 2.0 (Roche Diagnostics, Tokyo, Japan). The primer sets and conditions for PCR are described in the Supplemental Data (http://stroke.ahajournals.org).

Cell Culture
Human brain microvascular endothelial cells (HBECs, Cell Systems, Kirkland, WA) were serially cultured in 100-mm collagen type I coated dishes (IWAKI, Tokyo, Japan) using CS-C complete medium (Cell Systems). At subconfluence the medium was exchanged for serum-free medium containing growth factor but not phenol red to be in estrogen-free condition. HBECs were or were not treated with 10 ng/mL tumor necrosis factor (TNF)-α (Calbiochem, San Diego, CA) and incubated for 4 hours. The cells were then pretreated with pravastatin (10^−7, 10^−6, and 10^−5 mol/l) 1 hour before stimulation with TNF-α in the absence of estrogen. The vehicle control was kept in the complete medium with estrogen and not treated with TNF-α.

Western Blot Analysis
Protein was separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with primary antibodies against eNOS (BD Bioscience), ICAM (Santa Cruz), VCAM (R&D), and β-actin (Sigma, Steinheim, Germany). After incubation with horseradish peroxidase-conjugated secondary antibodies (American Bioscience, Buckinghamshire, UK), signals were detected by chemiluminescence using an Electrochemiluminescence (ECL) kit (GE Healthcare, Buckinghamshire, UK).

Statistical Analysis
Data were expressed as the mean±SD. Comparison of data was performed using the unpaired Student t test. The incidence of cerebral aneurysmal changes was analyzed with the Fisher exact test. The Mann-Whitney U test was used to compare the incidence of TUNEL-positive cells. P values of <0.05 were considered to indicate statistical significance.

Results
Progression of Cerebral Aneurysms in Rats Exposed to Renal Ligation, Hemodynamic Stress, and Estrogen Deficiency
To confirm progression of cerebral aneurysms, we monitored changes occurring at the left anterior cerebral artery-olfactory artery bifurcation for 24 weeks (Figure S1, http://stroke.ahajournals.org). In rats exposed to renal ligation, hemodynamic stress, and estrogen deficiency, aneurysmal changes progressed with time during the 24-week postoperative observation period (Figure 1). Endothelial damage (stage 1) was observed in 92% of rats within 6 weeks
and the incidence of cerebral aneurysms (stage 2 and stage 3) gradually increased with time (60% and 23% at 12 weeks, and 68% and 47% at 24 weeks), indicating that the aneurysms progressed until 24 weeks. However, none of the rats suffered SAH during 24-week observation period.

Bidirectional Effects of a Hydrophilic Statin on Cerebral Aneurysms

To examine the preventive and therapeutic effects of statins on cerebral aneurysms, we started pravastatin treatment 2 weeks before OVX (Figure S1, Exp. 2, http://stroke.ahajournals.org). Rats treated with 25 mg/kg per day or 50 mg/kg per day pravastatin (P-25 and P-50) had a higher incidence of cerebral aneurysms, especially saccular aneurysms, than did VC rats (Table S1, http://stroke.ahajournals.org; Figure 2A). More surprisingly, some suffered SAH (Figure 3A-3C). The site of SAH was not restricted to the anterior cerebral artery-olfactory artery bifurcation. As these results contradicted findings of others that statins are vasoprotective, we compared the effects of low-dose (5 mg/kg per day) pravastatin (P-5) and lipophilic simvastatin (Sim-5; Figure 2B). P-5 rats had a low incidence of endothelial damage and cerebral aneurysms, although the rate of saccular aneurysms was almost the same as in the VC group. In contrast, Sim-5 rats manifested a significantly higher incidence of cerebral aneurysm than did VC rats. These findings suggest that statins may exert different effects on cerebral aneurysms depending on the type of statin and dosage.

At High, But Not at Low Doses, a Hydrophilic Statin Induces Vascular Apoptosis in the Aneurysmal Wall, as Does a Lipophilic Statin

By inducing apoptosis in SMC, statins inhibit remodeling and prevent restenosis in the atherosclerotic vascular wall.8 To elucidate the mechanisms underlying the aggravation by statins, we focused on apoptosis in the vascular wall. As shown in Figure 4A and 4B, compared with VC rats, cleaved caspase-3 levels and TUNEL-positive cells were increased in the aneurysmal wall of P-50 and Sim-5 rats. In contrast, in P-5 rats, caspase-3 cells and TUNEL-positive cells were scarce, indicating that at high, but not at low doses, hydrophilic statin, like lipophilic statin, induces vascular apoptosis.
in the aneurysmal wall. Many caspase-3 positive cells were seen primarily in the medial layer of the aneurysmal wall and in some of the intimal layer (Figure 2A). However, there was no significant difference in the mRNA level of Bax, Bcl-2, and Bcl-xl among the 5 groups (data not shown).

Effect of Statins on the mRNA Level of Smooth Muscle α-Actin, Nox4, and Rac1

In cerebral aneurysms, thinning of the medial layer is frequently observed. To examine whether statins affect smooth muscle α-actin, we analyzed the α-actin mRNA level. In P-50 and Sim-5 rats, the mRNA level of α-actin was significantly lower than in VC rats (Figure 5A), suggesting that downregulation of α-actin contributes to the promotion of aneurysm formation. Most studies have demonstrated that statins ameliorate oxidative stress; however, some reported their potential pro-oxidant effects. We further examined whether statins affect the mRNA level of the nicotinamide adenine dinucleotide phosphate oxidase subunits Nox4 and Rac1. In agreement with our earlier findings, the mRNA level of these molecules was significantly higher in VC than in sham-operated rats (Figure 5A). Notably, the mRNA level of Nox4 in the Sim-5 group was dramatically higher than in the VC group, suggesting that simvastatin increases oxidative stress in cerebral vascular walls under some conditions. Neither statin had an effect on the Rac1 mRNA level.

A Hydrophilic Statin Protects Against Endothelial Injury

In earlier immunohistochemical studies, we demonstrated that while eNOS expression was obscure in the aneurysmal wall, the expression of angiotensin II, ICAM-1, and VCAM-1 was increased in the intima. Furthermore, the mRNA level of TNF-α, ICAM-1, and VCAM-1 was significantly higher in VC- than in sham-operated rats, suggesting that the pathogenesis of cerebral aneurysm is associated with endothelial dysfunction and inflammation. To confirm the vasoprotective effects of low-dose hydrophilic statin, we performed in vitro study using HBECs. As TNF-α is thought to play an essential role in cerebral aneurysm formation and in regulating ICAM-1 and VCAM-1, we examined the effects of statin on the expression of these molecules in TNF-α-stimulated HBECs. Under TNF-α stimulation in the absence of estrogen, the expression of eNOS was reduced, and ICAM-1 and VCAM-1 were increased (Figure 6A and 6B). In contrast, eNOS was increased and ICAM-1 and VCAM-1 decreased by pravastatin in a dose-dependent manner, indicating that pravastatin may exert protective effects against endothelial dysfunction and inflammation.

Discussion

We provide new evidence that statins exert both beneficial and deleterious effects on cerebral aneurysms, and that the observed aggravation was associated with vascular apoptosis. Statins, through their multiple pleiotropic actions, are generally thought to exert beneficial effects on the vasculature, protecting against restenosis and the development of atherosclerosis. As others and we found, formation and progression of cerebral aneurysms was associated with vascular inflammation and oxidative stress.5–7,14 We initially hypothesized that statins inhibit aneurysm formation through their anti-inflammatory and antioxidative stress effects. Unexpectedly,
Figure 4. A, Representative elastica van Gieson staining (EvG), immunohistochemical staining for the active cleaved subunits of caspase-3 (green) and double staining for caspase-3 (green) and smooth muscle α-actin (red), and terminal deoxyribonucleotidyl transferase (TUNEL) staining (green). B, TUNEL-positive cells per 150-μm² field around the aneurysm were counted (control, n=6; P-5, n=7; P-50, n=7; Sim-5, n=7; sham, n=6). Data (mean±SD) were analyzed by the Mann-Whitney U test. *P<0.05; †P<0.01 vs VC. VC indicates vehicle control; SD, standard deviation.
pravastatin exerted different effects on cerebral aneurysms; in rats treated with low doses, endothelial damage and aneurysm formation were reduced, while at higher doses it promoted formation of cerebral aneurysms. More notably, at the higher doses it induced aneurysmal rupture. Simvastatin promoted the formation of aneurysms even at a low dose. The progression of cerebral aneurysms was associated with an increase in apoptotic cells and downregulation of α-actin in the aneurysmal wall. Aoki et al. demonstrated the usefulness of statins (simvastatin and pitavastatin) in a male rat cerebral aneurysm model, and atorvastatin markedly attenuated neointimal formation in balloon-injured vessels in OVX female, but not in male, rats. These divergent results suggest that statins exert both beneficial and detrimental effects on the wall of cerebral aneurysms, and that these effects differ depending on the type of statin, their dosage, and on physiological conditions, including those related to sex differences.

Statins have been shown to inactivate p-21 RhoA protein via inhibition of its prenylation and subsequent downregulation of the expression of antiapoptotic Bcl-2 protein, or by stimulating the expression of TNFα receptor; this thereby potentiates TNFα-mediated apoptosis. These data support our results. As the apoptotic cells were focally localized in the aneurysmal walls, it may be impossible to detect differences with respect to the downregulation of mRNA levels of the apoptosis-related molecules; these are limited to the aneurysmal site, among the statin-treated groups with aneurysm at the anterior cerebral artery-olfactory artery bifurcation. Another possible mechanistic explanation for these findings is post-translational modification of statin-induced apoptosis.

The statin-induced apoptosis of vascular cells may be attributable to reduced synthesis of important intermediates involved in the post-translational prenylation of several proteins (Ras, Rho, Rac). Although we did not study the effects of statins on prenylation, the observed downregulation of α-actin in rats treated with high-dose pravastatin or low-dose simvastatin suggests a defect in the generation of VSMC in the presence of these statins in an apoptosis-dependent or -independent manner.

Both lipophilic and hydrophilic statins are efficient low-density lipoprotein (LDL)-lowering substances that decrease the occurrence of cardiovascular disease. Interestingly, only lipophilic statins have been shown to enhance VSMC apoptosis, even in the presence of survival factors. In particular, simvastatin and atorvastatin potentiated endothelial cell apoptosis and VSMC apoptosis. Conversely, hydrophilic rosuvastatin attenuated endothelial cell apoptosis in humans. Pravastatin suppressed VSMC apoptosis in both human and animal studies. This discrepancy is thought to be attributable to the absence or presence of drug hepatoselectivity. However, according to Weiss et al., who exposed human VSMC for short periods to high doses of pravastatin, induction of apoptosis was significant. Their findings support ours that pravastatin at high, but not at low doses, and simvastatin at low doses, enhance induction of apoptosis in the aneurysm. It is not known whether tissue levels of statins achieved in vivo approximate the level reported to enhance apoptosis in vitro, and whether long-
term and repeated exposure to different concentrations of statins affects apoptotic signals in vascular cells.23 We recently demonstrated that endothelial tight junction proteins were downregulated in cerebral aneurysm walls, and suggested that the degradation of tight junctions facilitates macrophage infiltration into the vascular wall.4 Our in vitro study showed protection from endothelial injury by pravastatin. However, once the endothelium was injured, even hydrophilic statins may be able to permeate endothelial cells and VSMC.

Chronic statin treatment increases levels of the tumor suppressor phosphatase and the tensin homolog deleted on chromosome X, suggesting an association with a proapoptotic effect. Thus, the effects of statins on vascular cells vary not only according to dose, but also to the administration period.

Whether vascular cell apoptosis induced by statins is beneficial or detrimental may depend on the disease. VSMC apoptosis plays a significant role in the control of neointimal thickening27 and in prevention of postangioplasty restenosis or venous graft occlusion.8,18 In advanced disease stages, the rate of VSMC apoptosis is augmented; this may contribute to plaque rupture and increased thrombogenicity, resulting in an increase in the incidence of acute coronary events.28 Cerebral aneurysms involve outward remodeling, and the aneurysmal wall tends to be thin rather than thick. As apoptosis was associated with aneurysmal growth and rupture, the proapoptotic effects of statins would be detrimental in cerebral aneurysms.

In the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) trial,29 the administration of high-dose atorvastatin resulted in a significant reduction in the number of ischemic strokes, but in an increase in hemorrhagic strokes. Meier et al30 reported that prior statin use, but not cholesterol levels at the time of admission, is associated with a higher incidence of intracranial hemorrhage after intra-arterial thrombolysis, without impacting the outcome. The administration of statin to patients discharged after suffering intracranial hemorrhage has increased modestly.31 The clinical results of the statin effects on vascular cell apoptosis appears to depend on both cell type (ie, endothelial, neuronal, or glial cells) and the existing balance between survival- and death-promoting factors. A further elucidation of this balance may explain clinical trials that reported significant benefits from statin use, but also undesired effects.

Statins were found to exert beneficial pleiotropic effects on the vasculature, but also to induce vascular cell apoptosis. As the regulation of vascular cell apoptosis is complex and involves multiple interacting molecular pathways, more detailed assessments are required to determine therapeutic usage of statins safe in patients with cerebral aneurysms.

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Disclosures
None.

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Materials and Methods

Induction of Experimental Cerebral Aneurysms

All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Animals of the American Physiological Society and approved by the Animal Care Committee of the University of Tokushima. Cerebral aneurysms were induced as described by Jamous et al.\textsuperscript{1, 2} All procedure were performed with the rats under isofluorane (2-4\%) inhalation anesthesia. Their blood pressure was measured by the tail-cuff, auto-pickup method. For aneurysm induction, 7-week-old female Sprague-Dawley rats were subjected to ligation of the left common carotid artery and the bilateral posterior renal arteries. One week later, we substituted their drinking water with a 1.0\% saline solution; one month post-ligation they underwent bilateral oophorectomy (OVX). We performed 4 experiments to examine progressive cerebral aneurysmal changes (Supplemental Figure. S1), to determine whether these changes can be prevented by statin treatment, and to assess the effect of coenzyme Q\textsubscript{10}, administered in combination with a statin, on cerebral aneurysm progression. As we previously observed aneurysmal changes primarily at the left anterior cerebral artery - olfactory artery (ACA- OA) bifurcation,\textsuperscript{3} we assessed changes at this site. For morphological assessments of cerebral aneurysms we used corrosion casts.

Experiment 1: Time course of aneurysmal changes at the left ACA-OA bifurcation

Rats exposed to renal ligation, hemodynamic stress, and estrogen deficiency were sacrificed 3-, 6-, 12-, and 24 weeks later for morphological assessments.

Experiment 2: Effects of high-dose pravastatin on rat cerebral aneurysms

Elsewhere we recently demonstrated that estrogen deficiency induces endothelial dysfunction in our rat aneurysm model.\textsuperscript{4} To examine whether pravastatin prevents the formation of aneurysms by improving endothelial dysfunction, we began treating rats with the drug 2 weeks before OVX. They were randomly divided into a vehicle control (VC) group (n=8) that received 5\% Arabic gum solution and 25- and 50 mg/kg/day pravastatin groups (P-25 and P-50 group, n=12 each); the drug was administered for a total of 14 weeks by gavage feeding (supplemental Figure S1).

Pravastatin was a gift by Sankyo Co. Ltd. (Tokyo, Japan). Twelve weeks after OVX the rats were euthanized as described below and samples for vascular corrosion casts were prepared.

Experiment 3: Comparison of the effect of low-dose pravastatin and simvastatin on aneurysm formation

Rats were randomly assigned to 3 groups (supplemental Figure S1) and received 5\% arabic gum solution (VC group, n=8), low-dose pravastatin (5 mg/kg/day,
P-5, n=12), or simvastatin in 5% Arabic gum solution (5 mg/kg/day, Sim-5 group, n=13) for 14 weeks. Simvastatin was purchased from Wako (Tokyo, Japan).

**Experiment 4:**

Statins block the production of farnesyl pyrophosphate, an intermediate in the synthesis of ubiquinone or coenzyme Q$_{10}$ (CoQ$_{10}$). The statin-induced reduction in CoQ$_{10}$ has been documented in animal and human studies.$^5$ We determined the formation and progression of cerebral aneurysms in rats treated with 50 mg/kg pravastatin alone (P-50 group, n=7) or with pravastatin plus CoQ$_{10}$ (15 mg/kg/day, CoQ$_{10}$ group, n=12).

**Subarachnoid hemorrhage (SAH) symptoms**

Due to the unavailability of CT instrumentation we were unable to confirm aneurysmal rupture on scans. We recorded the occurrence of seizures, bleeding from the nose, respiration changes, coma, gait disturbance, and death without preceding symptoms. When stroke symptoms were observed and it appeared that death was imminent, the rat was anesthetized deeply for tissue sampling. The brain was removed and examined for signs of SAH.

**Preparation and study of corrosion casts**

Vascular corrosion casts were prepared as previously described.$^3$ Briefly, the rats were transcardially perfused with heparinized phosphate-buffered saline (PBS), followed by Batoson’s No 17 plastic (Polyscience, PA, USA). The left ACA-OA bifurcation was inspected at 3kV under a scanning electron microscope (SEM) (VE8800, Keyence, Osaka, Japan) and changes were recorded as normal (stage 0), endothelial damage (stage 1), moderate protrusion (stage 2), and saccular aneurysm (stage 3). Morphological changes at the bifurcation were evaluated by 3 blinded observers and staged as described elsewhere.$^1$-$^4$ Normal bifurcations were recorded as stage 0 (no arterial dilation or irregular cell shapes or alignments). Stage 1 is characterized by a roughened apical intimal pad with irregularly-shaped endothelial cell imprints. In Stage 2 there is shallow fusiform elevation of the apical intimal pad, which is covered by abnormal endothelial cell imprints. In Stage 3, a well-developed saccular aneurysm is present.

**Immunohistochemistry and TUNEL staining**

Sham-operated- (n=6), VC- (n=6), P-5 (n=7), P-50 (n=7), and Sim-5 rats (n=7) at 12 weeks after OVX (at 23 weeks of age) were used for immunohistochemical study. After fixing, frozen sections were blocked and incubated with primary- and secondary antibodies (Alexa Fluor594 and 488 donkey anti-mouse-, donkey anti-goat-, or goat anti-rabbit IgG; Molecular Probes, Eugene, Oregon, USA). Primary polyclonal
antibodies against cleaved caspase-3 and monoclonal antibody against anti-smooth muscle α actin (Laboratory Vision, California, USA) were used. Sections from each sample were stained with elastica van Giesson (EvG) stain to detect the formation of cerebral aneurysms.

To assess apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling (TUNEL) staining was performed using the Apop Tag kit (S7100, Chemicon, CA, USA) according to the manufacturer’s protocol. The number of TUNEL-positive cells in 150 x 150-μm fields around the aneurysm was recorded.

Quantitative real-time PCR (qRT-PCR)

Samples from sham-operated- (n=6), VC- (n=6), P-5- (n=7), P-50- (n=7), and Sim-5 rats (n=7), were subjected to quantitative real-time PCR (qRT-PCR). At 12 weeks after OVX, the rats were euthanized as described above. The left ACA-OA bifurcation was isolated and total RNA was extracted using the EZ1 RNA Universal Tissue Kit (QIAGEN, Tokyo, Japan) and a MagNA lyser (Roche, Tokyo, Japan). Extracted RNA was treated with DNase (DNA-free; Ambion, Austin, TX) to remove genomic DNA. For reverse transcription of total RNA to cDNA we used the transcriptor first-strand cDNA synthesis kit (Roche). qRT-PCR of each sample was on a LightCycler 2.0 (Roche Diagnostics, Tokyo, Japan). LightCycler FastStart DNA master hybridization probes (Roche) were used for NOX4, Rac1, and GAPDH. Primers and probe sets for Bcl-2 were from Roche and used according to the manufacturer’s directions. The other primers were:

5’-acactctactggatgactgaa-3’/5’-tctgtatcccatctgttgact-3’ for NOX4, and

5’-gaagctgactcccactactc-3’/5’-cagcaggcattttcttccc-3’ for Rac1. The fluorescein/red-640 probes were:

5’-agacatccatctccctggcttgctgacac-3’/5’-tgctctagttgctgactcataaagatggtgccaa-3’ for NOX4, and

5’-ctgttctgttacttggcacttgctgtg-3’/5’-gacactccaggtatgtgacacagtctc-3’ for Rac1. The PCR conditions were 95°C for 10 min followed by 50 cycles at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 7 sec. Fluorescence measurements were taken at the end of the annealing phase. FastStart DNA master plus SYBR Green I (Roche) was used for Bax, Bcl-xl, and smooth muscle α-actin. The primers were

5’-cactaaagtgcccagctgtgatc-3’/5’-ttccagatgttgacagag-3’ for Bax,

5’-gcgttgtgtgacttcctc-3’/5’-gggttccctttcctggtc-3’ for Bcl-xl, and

5’-cagcactcaagactgtgacag-3’/5’-ggggtgtgactccaaacat-3’ for smooth muscle α-actin. The PCR conditions were 95°C for 10 min followed by 50 cycles at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 8 sec. We subjected samples from each group of rats to 2 independent qRT-PCR assays. GAPDH was the internal control.
Cell culture

Human brain microvascular endothelial cells (HBECs, Cell Systems, Kirkland, WA) were serially cultured in 100-mm collagen type I coated dishes (IWAKI, Tokyo, Japan) using CS-C complete medium (Cell Systems). Passages between 4 and 7 were used in the experiments. Each experiment was started at cell subconfluency. At subconfluence the medium was exchanged for serum-free medium containing growth factor but not phenol red to be in estrogen-free condition. HBECs were or were not treated with 10 ng/mL TNF-α (Calbiochem, San Diego, CA) and incubated for 4 hr. The cells were then pretreated with pravastatin (10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/l) 1 hr before stimulation with TNF-α in the absence of estrogen. The vehicle control was kept in the complete medium with estrogen and not treated with TNF-α.

Western blot analysis

Total protein in the cells was measured with the BCA protein kit (Pierce, Rockford, USA). Protein was separated by 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) solution-Tween 20 and then incubated with primary antibodies against eNOS (1:1000, BD Bioscience), ICAM (1:500, Santa Cruz), VCAM (1:200, R&D), and β-actin (1:100000, Sigma, Steinheim, Germany). After incubation with horseradish peroxidase-conjugated secondary antibodies (American Bioscience, Buckinghamshire, UK), signals were detected by chemiluminescence using an ECL kit (GE Healthcare, Buckinghamshire, UK). Images were analyzed with Image J software and normalized by setting the densitometry of the control sample to 1.0.

Statistical analysis

Data were expressed as the mean ± SD. Comparison of data was performed using the unpaired Student’s t test. The incidence of cerebral aneurysmal changes was analyzed with the Fisher exact test. The Mann-Whitney-U test was used to compare the incidence of TUNEL-positive cells. P values of <0.05 were considered to indicate statistical significance.
References


Supplemental Figure.S1: The time course of the experiment.

**Exp.1**
- RL
- CCAL
- OVX
- 3W
- 6W
- sacrifice
- saline
- 4W
- 3W
- 12W

**Exp.2**
- RL
- CCAL
- OVX
- 12W
- sacrifice
- saline
- 2W
- 12W
- Pravastatin 25mg/kg (n=12) or Pravastatin 50mg/kg (n=12)
- Vehicle control; 5% arabic gum (n=8)

**Exp.3**
- RL
- CCAL
- OVX
- 12W
- sacrifice
- saline
- 2W
- 12W
- Pravastatin 5 mg/kg (n=12) or simvastatin 5 mg/kg (n=13)
- Vehicle control; 5% arabic gum (n=8)

**Exp.4**
- RL
- CCAL
- OVX
- 12W
- sacrifice
- saline
- 2W
- 12W
- Pravastatin 50mg/kg (n=7) or Pravastatin 50 mg/kg + CoQ 10 (n=12)
- Vehicle control; 5% arabic gum (n=8)

Abbreviation: CCAL, right common carotid ligation; OVX, oophrectomy; RL, renal posterior artery ligation.
Supplemental Table S1: The incidence of aneurysmal changes in rats treated with pravastatin or simvastatin.

<table>
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<td>4</td>
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<td>(33%)</td>
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<td>2</td>
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<td>(17%)</td>
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<td>(16%)</td>
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<td>(8%)</td>
<td>(31%)</td>
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</table>

Abbreviation: P, pravastatin; Sim, simvastatin.
*p <0.05 vs VC by Fisher’s exact test.