Statins Induce Angiogenesis, Neurogenesis, and Synaptogenesis after Stroke

Jieli Chen, MD,1 Zheng Gang Zhang, MD, PhD,1 Yi Li, MD,1 Ying Wang, MD,1 Lei Wang, MD,1 Hao Jiang, PhD,1 Chenling Zhang, BS,1 Mei Lu, PhD,2 Mark Katakowski, BS,1 Carolyn S. Feldkamp, PhD,3 and Michael Chopp, PhD1,4

We demonstrate that the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors atorvastatin and simvastatin enhance functional outcome and induce brain plasticity when administered after stroke to rats. With atorvastatin treatment initiated 1 day after stroke, animals exhibited significant increases in vascular endothelial growth factor, cyclic guanosine monophosphate, angiogenesis, endogenous cell proliferation and neurogenesis, and an increase in the synaptic protein, synaptophysin. Atorvastatin-induced angiogenesis in a tube formation assay was reduced by an antibody against the vascular endothelial growth factor receptor 2 (FIK-1) and by the nitric oxide synthase inhibitor, N-mono-methyl-L-arginine (L-NAME). Atorvastatin also induced phosphorylation of Akt and Erk in cultured primary cortical neurons. These data indicate that atorvastatin induced brain plasticity and has neurorestorative activity after experimental stroke.

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The 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) reduce synthesis of cholesterol and isoprenoids, molecules that modulate diverse cell function.1 Statin therapy has pleiotropic effects, including protection against Alzheimer’s disease and neuroprotection.2 Therapy with statins modulates endothelial function and preserves blood flow to tissue exposed to an ischemic insult, by targeting the vascular wall, inflammatory cells and platelets, and extravascular tissues (brain parenchyma).2 Statins also induce angiogenesis in ischemic limbs of normocholesterolemic rabbits,3 upregulate endothelial nitric oxide synthase (NOS), and enhance cerebral blood flow (CBF).2

Although statins have shown neuroprotective benefits in neurological injury and disease, there are no studies to date that test the effects of statins on restoration of function after neural injury. In this study, we demonstrate that widely used statins (atorvastatin and simvastatin) when administered at 1 day after stroke is highly effective in reducing neurological deficits in a rat model of middle cerebral artery occlusion (MCAo). These benefits appear to be mediated by statin-induced amplification of angiogenesis, neurogenesis, and synaptogenesis.

Materials and Methods

Animal Middle Cerebral Artery Occlusion Model

Adult male Wistar rats weighing 270 to 300gm were used in all our experiments. We induced 2 hours of MCAo using a method of intraluminal vascular occlusion modified in our laboratory.4

Experimental Groups

Experimental groups consist of rats subjected to 2 hours of MCAo ($n = 48$). Twenty-four hours after MCAo, rats were fed atorvastatin (calcium, Pfizer, Groton, CT), dissolved in saline, 1mg/kg ($n = 12$), 3mg/kg ($n = 12$), or 8mg/kg ($n = 12$) daily for 7 days. Rats subjected to MCAo and fed water without atorvastatin served as a control group ($n = 12$). All animals received daily intraperitoneal injections of bromodeoxyuridine (BrdU; a thymidine analog, which labels newly synthesized DNA, 100mg/kg; Sigma, St. Louis, MO) starting at 24 hours after MCAo and subsequently for 13 consecutive days for identification of endothelial cell and endogenous subventricular zone (SVZ) and dentate gyrus (DG) cell proliferation. All animals were killed at 14 days after...
MCAo. To test whether the effect of statin treatment of stroke is specific to atorvastatin, we treated rats with simvastatin (1mg/kg, n = 8) using the identical treatment protocol used for atorvastatin. Atorvastatin and simvastatin (Merck, Somerset, NJ) were purchased from Henry Ford Hospital pharmacy.

Functional Tests
Adhesive-removal somatosensory test6,6 and a modified neurological severity score6,7 evaluation were performed before MCAo and at 1, 7, and 14 days after MCAo by an investigator who was blinded to the experimental groups.

Histological and Immunohistochemical Assessment
At 14 days after MCAo, animal (n = 5/group) brains were embedded in paraffin. Lesion volume was measured using the Global Lab Image analysis system (Data Translation, Malboro, MA) and presented as a volume percentage of the lesion compared with the contralateral hemisphere.

IMMUNOHISTOCHEMISTRY. BrdU, von Willebrand factor (dilution, 1:400; Dako, Carpinteria, CA) and synaptophysin (monoclonal antibody, clone SY 38, 1:40; Boehringer (dilution, 1:400; Dako, Carpinteria, CA) and synaptophysin (monoclonal antibody, clone SY 38, 1:40; Boehringer Mannheim Biochemica, Indianapolis, IN) immunostaining were performed, as previously described.6,7 To determine whether BrdU-immunoreactive cells express neural phenotype, we used double immunohistochemical staining to identify BrdU (1:100; Boehringer Mannheim, Indianapolis, IN) with the progenitor neuronal marker, TUJ1 (1:400, Monoclonal Anti–B–Tubulin Isotype III, Sigma). Using double immunohistochemical staining, we counted a total of 500 BrdU-positive cells in the SVZ per animal to obtain the percentage of BrdU-reactive cells colocalized with TUJ1.

QUANTIFICATION. BrdU-immunostained sections were digitized using a ×40 objective (Olympus BX40) via the MCID computer imaging analysis system (Imaging Research, St. Catharines, Ontario, Canada). Cells within a total of 20 enlarged and thin-walled vessels located in the boundary area of the ischemic lesion were counted in each section. BrdU-reactive cell numbers were counted in the SVZ and DG of the ischemic hemisphere. Five sections and eight views in each section were counted per rat with the number of BrdU-reactive cells averaged. Data are presented as the number of BrdU-reactive cells per section.

For measurement of vascular density and perimeters, each vWF-immunostained coronal section was digitized using a ×20 objective via the MCID computer imaging analysis system. The numbers of vessels and their perimeters were counted throughout each field of view. The total number of vessels was divided by the total tissue area to determine vascular density.

For semiquantification of synaptophysin immunoreactivity, five immunostained coronal sections and eight fields of view from the ischemic penumbra (cortex and striatum) and contralateral hemisphere in each section were digitized under a ×20 objective. The optical density was measured. All values of density are presented as a percentage compared with the contralateral homologous region on the same section.

Vascular Endothelial Growth Factor Detection
Vascular endothelial growth factor detection (VEGF) was measured using a VEGF ELISA kit (mouse VEGF Immunoassay, catalog no. MIVM00, Quantikine M; R & D Systems, Minneapolis, MN).7,10 Brain extract from control MCAo and atorvastatin- (1mg/kg) treated animals (n = 6/group) was obtained from the ischemic boundary zone at 14 days after MCAo.

Capillary-like Tube Formation Assay
Formation of capillary-like tubes in mouse brain–derived endothelial cells was measured.11,12 Mouse brain–derived microvessel endothelial cells (2 × 10^4 cells) in 1.5ml culture regular medium or atorvastatin were added to each dish. These cells were incubated in (1) regular cell culture medium (Dulbecco’s modified Eagle medium) for control; atorvastatin or (2) 0.01µM, or (3) 0.1µM, or (4) 0.5µM, or (5) 1.0µM, or (6) 10µM, or (7) 0.5µM with 10µg/ml anti–mouse neutralizing antibody to VEGFR-2 (DC101; ImClone System, New York, NY); (8) 0.5µM atorvastatin with 5mM NOS inhibitor N-mono-methyl-1-arginine (L-NAME, Sigma); (9) 0.5µM atorvastatin with 10µg/ml anti–mouse neutralizing antibody to VEGFR-2 (DC101; Imclone System) and 5mM L-NAME. All assays were performed in triplicate and quantitated.

Corneal Assay for Angiogenesis
To test effect of atorvastatin on induction of angiogenesis in an in vivo system, we performed a corneal assay for angiogenesis (n = 6). Mixed atorvastatin (2µl, 10µg) with 12% hydron (2µl) was inserted 2mm away from the limbus margin of the cornea of the right eye.13 Twelve percent hydron (4µl) alone was inserted into left eye for control. Corneas were digitized under a ×2.5 objective for measurement of vessel length extension (in millimeters) and density using a video camera interfaced with MCID image analysis system.

Cyclic Guanosine Monophosphate Measurement in Brain Tissue
Levels of cyclic guanosine monophosphate (cGMP) in the ipsilateral hemisphere of ischemic brain with (1mg/kg for 7 days) or without atorvastatin treatment after stroke in rats were measured (n = 6/group). The animals were killed at 7 days after stroke. cGMP was measured using a commercially available low pH Immunoassay kit (R & D Systems).14 Values for cGMP were standardized by total protein (micromolars per milligrams protein).

Signaling Pathway of Atorvastatin-treated Cultured Cortical Neurons
Phosphorylated Akt and Erk were measured using Western blot analysis.15 Cultured primary cortical neurons were prepared from embryonic day 17 Wistar rats and pretreated with or without the phosphatidylinositol 3’ kinase (PI3K) inhibitor, LY294002 (20µM; Calbiochem, San Diego, CA), followed by treatment with 0.5µM of atorvastatin. Lysates were probed with anti-phospho-Akt (Ser473, Cell Signaling Technology, Beverly, MA) and anti-phospho-Erk1 and Erk2 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies and detected using a SuperSignal protein detection kit (Pierce,
New York, NY) with an horseradish peroxidase–conjugated secondary antibody. Three independent experiments were performed. The intensity of the bands was measured using Scion image analysis (Scion Cooperation, Frederick, MD).

Cholesterol, Blood Pressure, and Cerebral Blood Flow Measurements
Serum total cholesterol was quantified (n = 3/group). Systemic blood pressure (BP) (Protocol Systems; INS, Beaverton, OR; n = 3/group) and regional CBF (rCBF) was measured using laser-Doppler flowmetry,16 30 minutes before treatment to 3 hours after treatment with atorvastatin (n = 3/group). The data are presented as a percentage of before treatment baseline values.

Statistical Analysis
Given a lack of normality of the data, the generalized estimation equation approach was used for analysis of functional test scores and lesion volume. For each outcome, one-way analysis of variance was used to test for VEGF, neurogenesis, and angiogenesis between groups with and without treatment of atorvastatin at the significance level of 0.05. Data are presented as mean ± standard error.

Results
Neurological Outcome and Lesion Volume
All animals survived elective surgery. Compared with the control treated group, rats treated with 1 and 3mg/kg atorvastatin and 1mg/kg simvastatin had significantly (p < 0.05) improved functional recovery, with no functional benefit detected with 8mg/kg atorvastatin (Table). No significant differences of ischemic lesion volumes in atorvastatin and simvastatin-treated groups (29 ± 7.0% for 1mg/kg atorvastatin; 28.1 ± 4.9% for 3mg/kg atorvastatin; 42.3 ± 6.9% for 8mg/kg atorvastatin; 29.4±4.0% for 1mg/kg simvastatin) were detected compared with control treated rats (34.7 ± 5.6 %), respectively.

Endothelial Cell Proliferation and Angiogenesis
Enlarged and thin-walled vessels, containing BrdU-reactive endothelial cells indicative of angiogenesis,17 significantly increased in the ipsilateral hemisphere of the low-dose atorvastatin (1 and 3mg/kg) treatment group (Fig 1c, d), but not in the high-dose atorvastatin (8mg/kg, see Fig 1d) compared with the ipsilateral hemisphere of control animals (see Fig 1b, d) or the contralateral hemisphere of treated animals (see Fig 1a). Treatment with low-dose (1 and 3mg/kg) but not in high-dose (8mg/kg) atorvastatin (see Fig 1e, f) significantly (p < 0.05) increased vascular perimeter and density in the ipsilateral hemisphere compared with the control rats (see Fig 1e, f). These data indicate that low-dose atorvastatin enhances angiogenesis in ischemic brain.

Atorvastatin Promotes Vascular Endothelial Growth Factor Detection Secretion in the Ischemic Boundary Area
We tested whether atorvastatin evokes production of VEGF, a principal angiogenic factor,18 in ischemic brain. Using ELISA, we found that VEGF production within the ischemic boundary area at 14 days after stroke significantly increased in the low-dose atorvastatin (1mg/kg) treatment group (25.7 ± 2.7ng/ml) compared with the MCAo control group (10.5 ± 1.7ng/ml) and normal brain (13.4 ± 1.5ng/ml, p < 0.05).

Atorvastatin Promotes Corneal Neovascularization
As a complementary measurement of atorvastatin mediated angiogenesis, we used a corneal angiogenesis assay. Atorvastatin significantly augmented corneal neovascularization (see Fig 1h) compared with control animals (see Fig 1g). The total vascular lengths signif-

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*p < 0.05 compared with control MCAo group.
MCAo = middle cerebral artery occlusion; SE = standard error; NSS = neurological severity score.
significantly increased in the atorvastatin treatment group (29.9 ± 7.9mm/cornea) compared with control animals (1.5 ± 1.0mm/cornea).

**Vascular Endothelial Growth Factor Mediates Atorvastatin-induced Angiogenesis In Vitro**

Atorvastatin induced mouse brain-derived endothelial cells to form a network of capillary-like structures with numerous intercellular contacts (in 0.5µM atorvastatin, Fig 2c). Low concentrations of atorvastatin (0.01–1µM), but not a high concentration (10µM) significantly induced tube formation compared with control medium (see Fig 2a). Total capillary tube length significantly increased in medium containing atorvastatin (0.5µM, see Fig 2a, c) compared with the control medium (see Fig 2a, b). Both anti–VEGF receptor 2 (DC101; see Fig 2d, g) and NOS inhibitor L-NAME (see Fig 2e, g) significantly decreased atorvastatin-induced capillary tube formation compared with atorvastatin alone (p < 0.05, see Fig 2c, g). The combination of anti–VEGF receptor 2 (DC101) with L-NAME significantly inhibited atorvastatin-induced capillary tube formation (see Fig 2f, g) compared with atorvastatin alone (p < 0.05), and tube formation was significantly
reduced compared with atorvastatin with L-NAME ($p < 0.05$). These data suggest that both VEGF and NO facilitate atorvastatin-induced angiogenesis.

**Cell Proliferation in the Subventricular Zone and Dentate Gyrus**

BrdU-positive cells significantly increased in the ipsilateral DG (Fig 3b, c) and SVZ (see Fig 3e, f) in low-dose atorvastatin (1 and 3mg/kg) but not high-dose (8mg/kg, see Fig 3c, f) treated groups compared with the control treatment group (see Fig 3a, c for DG, see Fig 3d–f for SVZ). Using confocal imaging, we found that double staining immunohistochemistry shows that some BrdU-positive cells (see Fig 3g and i) colocalized with the developmental neuronal marker TUJ1 (see Fig 3h and i) in the low dose (1 and 3mg/kg) atorvastatin treatment group. The percentage of BrdU-reactive cells that express TUJ1 in the ipsilateral SVZ increased in low-dose atorvastatin-treated animals (1 and 3mg/kg; 25.4 ± 4.5%, 21.4 ± 2.1%, $p < 0.05$), but not in high-dose–treated animals (8mg/kg, 15 ± 5.8%) compared with control group (14.4 ± 6.2%).

**Effects of Atorvastatin on Ischemic Brain Cyclic Guanosine Monophosphate**

cGMP promotes neurogenesis.19 Statins act as NO donors20 and thereby may enhance NO-mediated increase in cGMP. To test this hypothesis, we measured cGMP in ischemic brain in both control and atorvastatin-treated rats at 8 days after stroke. Administration of low-dose (1mg/kg) atorvastatin at 24 hours after MCAo for 7 days significantly increased cGMP (3.9 ± 0.5 μmol/mg protein, $p < 0.05$) compared with the control group (2.3 ± 0.1 μmol/mg protein) in the ipsilateral hemisphere.

**Synaptophysin**

Functional benefit derived from treatment of stroke with atorvastatin suggests an effect of atorvastatin on synaptic plasticity. Synaptophysin is a marker for presynaptic plasticity and synaptogenesis.21 Figure 4 shows synaptophysin expression significantly increased in the low-dose (see Fig 4b, c) but not in high-dose atorvastatin treatment rats compared with control rats (see Fig 4a, c).

**Effect of Atorvastatin on Activation of Akt and Erk in Cultured Primary Cortical Neurons**

Statins activate PI3K/Akt and Ras/Erk pathways.22 Phosphorylation of Akt and Erk transduces cell survival signals and promotes synaptic plasticity.23–25 We therefore measured phosphorylation of Akt and Erk in primary cortical neurons. Incubation of atorvastatin (0.5μM) in cultured primary cortical neurons mark-
edly increased phosphorylation of Akt (265%, Fig 5a) and Erk (140%, see Fig 5b) compared with control (see Fig 5a, b). Pretreatment with LY294002, a stable PI3K inhibitor, significantly reduced atorvastatin-induced phosphorylation of Akt and Erk (75% and 2.7%, respectively; see Fig 5a, b). These data indicate that atorvastatin activates the PI3K/Akt and Erk pathways in cortical neurons.

**Serum Total Cholesterol, Blood Pressure, and Regional Cerebral Blood Flow**

Serum total cholesterol was not significantly different in any group (1mg/kg, 66.3 ± 4.9mg/dl; 3mg/kg, 71 ± 11.5mg/dl; 8mg/kg, 64 ± 10.1mg/dl; and control group, 76.4 ± 9.4mg/dl). This suggests that atorvastatin-induced brain plasticity is not dependent on the lowering of cholesterol. High-dose (8mg/kg) treatment with atorvastatin caused a significant reduction (p < 0.05) of systemic arterial BP levels at 1 hour after treatment (51 ± 10.6mm Hg) compared with levels obtained before treatment (85.0 ± 3.3mm Hg). BP was not decreased at 1 hour after low-dose (1 and 3mg/kg) atorvastatin administration (78.3 ± 4.7 and 81.6 ± 12.1mm Hg, respectively) compared with BP measured before treatment (84.3 ± 4.0 and 76.3 ± 6.3mm Hg, respectively). rCBF significantly increased in the ischemic hemisphere in low-dose atorvastatin (1mg/kg) treatment from 40 to 120 minutes after atorvastatin administration (range, 165 ± 22% to 130 ± 3.9%, p < 0.05) but not in high-dose (8mg/kg; range, 86.2 ± 9.4% to 115.3 ± 15.1%) treatment compared with pretreatment levels. No significant differences in rCBF were detected 2 hours after atorvastatin treatment among all groups.
Discussion

We demonstrate for the first time to our knowledge that a widely clinically used statin, atorvastatin, when administered at low dose to a rat 1 day after MCAo evokes significant improvement in functional neurological recovery. Concomitant with this neurological benefit is a significant induction of angiogenesis, cell proliferation in neurogenic zones within brain, and increased expression of a synaptic protein, synaptophysin. Treatment of stroke with simvastatin also provided significant functional benefit and no change in the volume of cerebral infarction. Thus, the therapeutic effect is not specific to atorvastatin.

Atorvastatin promotes neurological functional recovery in rats after stroke and induces angiogenesis, neurogenesis, and synaptogenesis in low-dose (1 and 3mg/kg) but not in high-dose (8mg/kg) treatment. Low, but not high, concentrations of atorvastatin induce capillary tube formation and promote angiogenesis. This dose-dependent response is consistent with in vitro data. HMG-CoA reductase inhibition decreases VEGF secretion in human endothelial cells at high statin dose.10 Endothelial cell proliferation, migration, and differentiation were enhanced at low concentrations, but significantly inhibited at high statin concentrations.10 The loss of functional benefit in animals treated with high-dose atorvastatin also may be attributed to a reduction of systemic arterial pressure. Hypotension after stroke exacerbates the ischemic lesion.26 These data suggest that high-dose atorvastatin may
have an adverse effect on the ischemic lesion and on functional recovery.

Increased angiogenesis promotes neurological functional recovery after stroke. Our data demonstrate that low-dose atorvastatin promotes endothelial cell proliferation and increased vessel perimeter and density, induces angiogenesis, increases CBF, and improves functional recovery. Statins may enhance recovery from cerebral ischemia through beneficial modulation of the brain vascular system and the promotion of angiogenesis.

Administration of atorvastatin significantly increased the number of BrdU-immunoreactive cells in the SVZ and DG, and some cells colocalized with TUJ1, suggesting that atorvastatin promotes neurogenesis in the adult brain after stroke. This enhanced neural progenitor production may play a role in neurological functional recovery.27 However, we have not demonstrated that these newly produced cells integrate into the cerebral architecture and are electrically active. Therefore, the data associating neurogenesis with functional recovery should be interpreted with caution.

Reduction of neurological deficits after stroke has been attributed to extensive synaptic and functional reorganization.28 Treatment with low-dose atorvastatin initiated 24 hours after MCAo significantly promotes synaptophysin expression in the ischemic boundary and thereby may improve functional outcome.

Activation of PI3K/Akt and Erk signaling in neurons plays a central role in controlling synaptic plasticity and memory and may be implicated in atorvastatin induction of angiogenesis, neurogenesis, and synaptogenesis. Activated Akt differentiates PC12 cells, accelerates motor axon regeneration in vivo, and increases axon caliber and branching in sensory neurons. p-Erk-2 fusion protein enters the nucleus and promotes axon elongation.24 We demonstrate that atorvastatin promotes p-Akt and p-Erk in primary cortical neurons. Phosphorylation of PI3K/Akt leads to posttranscriptional activation of endothelial NOS, which promotes NO production. NO activates soluble guanylyl cyclase leading to the formation of cGMP, as we have found. In the central nervous system, NO/cGMP signaling promotes synaptic plasticity, axonal outgrowth and neurogenesis, and angiogenesis during development and also in the mature animal.30–33 NO/cGMP regulates expression of transcriptional genes and some of these genes, such as the cAMP response element–binding protein, are involved in cellular survival, neurogenesis,31,32 differentiation, synaptogenesis, neurotransmitter release, and synaptic plasticity.33,34 Atorvastatin also promotes VEGF secretion in the ischemic brain. VEGF is an angiogenic factor and stimulates axon outgrowth.35 The significant inhibition of capillary tube formation by anti–VEGFR2 antibody and by L-NAME and the added inhibition of angiogenesis by L-NAME in combination with anti-VEGFR2 indicate that angiogenesis is promoted by both VEGF and NO with contributions from the PI3K/Akt pathway.36,37 Thus, the atorvastatin influenced PI3K/Akt/NO/cGMP and Ras/Erk pathways may be related to angiogenesis, neurogenesis, brain plasticity, and synaptogenesis.

In summary, we demonstrate that treatment of experimental stroke with low-dose atorvastatin at 24 hours after stroke significantly improves functional outcome. This functional benefit may be related to enhancement of angiogenesis, neurogenesis, and synaptogenesis after stroke.

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