Increase in phosphorylation of Akt and its downstream signaling targets and suppression of apoptosis by simvastatin after traumatic brain injury

Laboratory investigation

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Object. In their previous studies, the authors found that simvastatin treatment of traumatic brain injury (TBI) in rats had beneficial effects on spatial learning functions. In the current study they wanted to determine whether simvastatin suppressed neuronal cell apoptosis after TBI, and if so, they wanted to examine the underlying mechanisms of this process.

Methods. Saline or simvastatin (1 mg/kg) was administered orally to rats starting on Day 1 after TBI and then daily for 14 days. Modified Neurological Severity Scores were used to evaluate the sensory motor functional recovery. Rats were killed at 1, 3, 7, 14, and 35 days after treatment, and brain tissue was harvested for terminal deoxynucleotidyl nick-end labeling (TUNEL) staining, caspase-3 activity assay, and Western blot analysis.

Results. Simvastatin significantly decreased the modified Neurological Severity Scores from Days 7 to 35 after TBI, significantly reduced the number of TUNEL-positive cells at Day 3, suppressed the caspase-3 activity at Days 1 and 3 after TBI, and increased phosphorylation of Akt as well as Forkhead transcription factor 1, inhibitory-κB, and endothelial nitric oxide synthase, which are the downstream targets of the prosurvival Akt signaling protein.

Conclusions. These data suggested that simvastatin reduces the apoptosis in neuronal cells and improves the sensory motor function recovery after TBI. These beneficial effects of simvastatin may be mediated through activation of Akt, Forkhead transcription factor 1 and nuclear factor–κB signaling pathways, which suppress the activation of caspase-3 and apoptotic cell death, and thereby, lead to neuronal function recovery after TBI.

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Key Words • Akt • apoptosis • FOXO1 • IκB • rat • simvastatin • traumatic brain injury

Simvastatin is a member of the 3-hydroxy-3-methylglutaryl coenzyme A reductase family of drugs and recently has been described as promoting therapeutic benefit in pathological conditions other than hyperlipidemia.2 Our previous studies have demonstrated the beneficial effects of simvastatin on functional recovery and neurogenesis after TBI in rats,19,20 Statins increase synaptogenesis following neuronal hypoxia in a model of ischemic stroke,3 and they increase vascular endothelial growth factor, improve cerebral blood flow, and enhance brain plasticity.31 Chronic simvastatin treatment has been shown to be neuroprotective both in vivo14 and in vitro.35 Although the numerous, noncholesterol-related benefits of simvastatin have been identified, the mechanism underlying these effects has not been well defined. This is particularly true in the area of statin-induced neuroprotection, where simply lowering cholesterol levels does not appear to be the sole regulator of neuroprotection.

The phosphoinositide-3-kinase/Akt signaling pathway plays a crucial role in cell growth and cell survival. 
Activated Akt phosphorylates several downstream signaling target proteins, including Bad, glycogen synthase kinase 3–β, FOXOs, and IkB to control cell growth, cell survival, and protein synthesis. The FOXOs regulate the transcription of the cell cycle inhibitor p27 as well as the proapoptotic Fas ligand and the Bcl-2–like protein Bim. Phosphorylation of IkB leads to its dissociation and activation of NF-κB, which induces the expression of several genes that promote neuron survival, including those encoding manganese superoxide dismutase, inhibitor-of-apoptosis proteins, Akt, Bcl-2, and calbindin.

In this study we investigated the effects of simvastatin on apoptotic cell death after TBI. To examine a possible mechanism for the neuroprotective effects of simvastatin that subsequently enhanced the functional recovery, we focused on the Akt pathway, which has been implicated in the survival of neuronal cells.

**Methods**

**Animal Models**

In the present study we used a modified controlled cortical injury model of TBI in rats. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Adult male Wistar rats weighing 300–400 g were anesthetized intraperitoneally with 350 mg/kg body weight chloral hydrate. Rectal temperature was maintained at 37°C by using a feedback-regulated water heating pad. A controlled cortical injury device was used to induce injury. The rats were placed in a stereotactic frame. Two 10-mm-diameter craniotomies were performed adjacent to the central suture, midway between the lambda and the bregma. The second craniotomy allowed for lateral movement of cortical tissue. The dura mater was kept intact over the cortical tissue. The dura mater was kept intact over the cortical tissue. In the present study we used a modified controlled cortical injury model of TBI in rats. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

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**Experimental Groups**

Male Wistar rats were randomly divided into 3 groups. Rats in Group 1 (40 rats) were exposed to TBI and given saline orally 1 day later and consecutively for 14 days. Rats in Group 2 (40 rats) were subjected to TBI, and 1 day later simvastatin was administered orally at a dose of 1 mg/kg/day for 14 consecutive days. This dose was selected based on our previously reported study. Rats in Group 3 (8 rats) were subjected to sham surgery. Neurological functional evaluation was performed on Days 1, 3, 7, 14, and 35 after TBI. Rats in the first and second groups were killed at 1, 3, 7, 14, and 35 days after administration of simvastatin, and rats in the sham group were killed 1 day after TBI. Sixteen animals were killed at each time point; 8 rats were used for immunohistochemical analysis, and the other 8 rats were used for Western blot analysis.

**Neurological Functional Evaluation**

Neurological functional measurement was performed using an mNSS. The mNSS is a composite of the motor (muscle status and abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests. This test has been used in previous studies. Motor tests of the mNSS include 7 items with a maximum of 6 points. Sensory tests include 2 items with a maximum of 2 points. Beam Balance Tests have 7 items with a maximum score of 6. The last part of the mNSS includes the pinna, corneal, and startle reflexes and abnormal movements. Insult in the left hemisphere cortex of rats causes sensory and motor functional deficiency with elevated scores early after TBI.

**Tissue Preparation**

For immunostaining, rats were anesthetized intraperitoneally with chloral hydrate (350 mg/kg), and perfused transcardially with saline, followed by 4% paraformaldehyde. Brains were isolated, postfixed in 4% paraformaldehyde for 2 days at room temperature, and then processed for paraffin sectioning. A series of 10-μm-thick sections were cut with a microtome through each of 7 standard sections. For biochemical analysis, the ipsilateral and contralateral cortex and hippocampus tissues were dissected, frozen in liquid nitrogen, and stored at −80°C until use.

**The TUNEL Staining**

To identify cellular apoptosis, TUNEL was performed using an ApopTag peroxidase in situ apoptosis detection Kit (Chemicon). The paraffin-embedded coronal sections at the level of the hippocampus and dentate gyrus were selected and processed for TUNEL staining. After the digesting and quenching steps, equilibration buffer was applied directly to the sections, and working-strength TdT enzyme was then applied directly. A biotin-conjugated antidigoxigenin antibody was used. The sections were then incubated with biotinylated secondary antibody at 1:100 dilution for 45 minutes at room temperature and later with horseradish peroxidase–labeled streptavidin at 1:100 dilution for 45 minutes at room temperature. After a brief wash, 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml)/H2O2 (0.01%), a chromogen substrate, was incorporated. To quantify apoptotic neuronal cells in different areas, 10 consecutive fields were randomly selected and evaluated at 400× using an imaging analyzer. A blind-counting method was used to count TUNEL-positive cell numbers. For counting, images were obtained from the cortex, hippocampus, and dentate gyrus of the ipsilateral side of the injured animals. In each image we manually counted the TUNEL-positive cells from the boundary zone in the cortex, the CA3 region in the hippocampus, and the body and hilus of the dentate gyrus.

**Caspase-3 Activity Assay**

The activity of caspase-3 was determined using an Apo-Alert colorimetric caspase-3 assay kit (BD Biosciences Clontech). Brain cortex tissues in the boundary zone were collected and lysed in lysis buffer and equal amounts of lysates were used for caspase-3 activity assay, which was measured at a wavelength of 405 nm using the detection
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of chromophore pNA after its cleavage by caspase-3 from the labeled caspase-3 specific substrate, DEVD-pNA. The data are presented as pmoles of pNA per microgram of cell lysate per hour of incubation.

**Western Blot Analysis**

Rats in Groups 1 and 2 were killed at Days 1, 3, 7, 14, and 35 after administration of simvastatin (4 rats/time point/group). Brain tissues from the lesion boundary zone and the hippocampus were collected, washed once in 1× phosphate-buffered saline and lysed in lysis buffer (20 mM Tris pH 7.6, 100 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1% deoxycholic acid, 10% glycerol, 1 mM ethylenediamine tetraacetic acid, 1 mM NaVO₃, 50 mM NaF, cocktail I of protease inhibitors from Calbiochem). After sonication, supernatant protein was obtained by centrifugation at 13,000 G for 15 minutes at 4°C. The protein concentration of each sample was determined by bicinchoninic acid protein assay (Pierce). For immunoblotting, equal amounts of cell lysate were subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis on Novex Tris-Glycine precast gels (Invitrogen) and separated proteins were then electrotransferred to polyvinylidene fluoride membranes. Membranes were blocked with 2% I-Block (Applied Biosystems) in phosphate-buffered saline plus 0.1% Tween 20 for 1 hour at room temperature and then incubated with different primary antibodies overnight at 4°C. We used the following antibodies: anti–phosphorylated eNOS (Ser1177), anti–phosphorylated FOXO1 (Ser256), and anti–phosphorylated IkB (Ser32) (1:1000 dilution, Cell Signaling Technology); anti–phosphorylated Akt (Ser473) and anti-Actin (I-19) (1:1000; Santa Cruz Biotechnology). After washing, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (1:2500; Jackson ImmunoResearch Laboratories) in blocking buffer for 2 hours at room temperature. Specific proteins were visualized using SuperSignal West Pico chemiluminescence substrate system (Pierce). The intensity of the bands was measured using Scion image analysis (Scion Corp.).

**Statistical Analysis**

All data are presented as means ± SDs. Data were analyzed using a one-way analysis of variance. Differences were determined to be significant at a probability value < 0.05. All measurements were performed by observers blinded to individual treatments.

**Results**

**Simvastatin Enhances the Recovery of Sensorimotor Function After TBI**

Injury in the left hemisphere cortex of rats causes neurological functional deficits as measured by the mNSS. These rats presented with high scores on motor, sensory, and Beam Balance Tests on Day 1 after injury. Absent reflexes and abnormal movements were evident in rats with severe injury. On Day 3 after injury, recovery began, and this recovery persisted at all subsequent evaluation time points in both saline-treated and simvastatin-treated groups. Motor function tested by the mNSS recovered faster than sensory and beam balance functions. By Day 14 after injury, residual deficit scores were present mainly on the Beam Balance Tests and sensory (placing) test of the mNSS. The mNSS scores for the simvastatin-treated group were significantly decreased at Days 7 and 14 (6.2 ± 1.2 and 5.0 ± 0.6, respectively; *p < 0.05) after TBI when compared with the saline-treated groups (8.2 ± 0.9 and 7.1 ± 0.7, respectively; **p < 0.05) (Fig. 1).

**Simvastatin Reduces TUNEL-Positive Cells**

The TUNEL staining has been used extensively to identify cells with nuclear DNA fragmentation. Cells were scored as apoptotic when they were TUNEL-positive (brown staining in Fig. 2a–l) and showed nuclear chromatin condensation (Fig. 2a). In each hemisphere section of the sham-operated rats and in the contralateral hemisphere of ischemic rats, cells were almost negative for TUNEL staining, with only 0–3 TUNEL-positive cells. Those cells with nuclei not stained with TUNEL (TUNEL-negative, blue color in Fig. 2a–l) were visualized by counterstaining with hematoxylin. In contrast, in the ipsilateral hemisphere in the saline-treated control group, a large number of TUNEL-positive cells, which were mainly located in the ipsilateral boundary cortex, hippocampus, and dentate gyrus, were seen at 24 hours after TBI. Compared with the saline-treated group simvastatin reduced the number of TUNEL-positive cells (74 ± 6.8 vs 52 ± 4.6 in the lesion boundary zone, p < 0.05; 15 ± 4.5 vs 6 ± 2.1 in the hippocampus, p < 0.01) notable on Day 3 after treatment (Fig. 2o and m), demonstrating that simvastatin reduces apoptosis in the hippocampus and the lesion boundary zone after TBI.

**Fig. 1.** Graph showing the temporal profile of the mNSS after TBI in saline- or simvastatin-treated rats (8/group). The score was significantly decreased in the simvastatin-treated group compared with the saline-treated group from 7 days after treatment. These data demonstrate that simvastatin enhances the sensorimotor functional recovery after TBI. Data are represented as means ± SDs. *, p < 0.05; **, p < 0.01.
Simvastatin Reduces Apoptotic Cell Death Through Suppression of Caspase-3 Activity

Caspase-3 activity was measured to determine if simvastatin suppressed the TBI-induced apoptosis via the caspase cascade, which is the central protease in the cell death pathway. Colorimetric assay showed a significant decrease in caspase-3 activity at Days 1 (28.56 ± 3.9 vs 19.45 ± 2.9, p < 0.05) and 3 (64.86 ± 13.98 vs 24.15 ± 12.01, p < 0.01) after simvastatin treatment (Fig. 3), suggesting that simvastatin suppresses the cleavage of caspase-3.

Phosphorylation of Akt, eNOS, FOXO1, and IκB in the Ipsilateral Hemisphere Following Treatment With Simvastatin After TBI

We have previously shown that simvastatin signifi-
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![Fig. 3. Bar graphs showing the activity of caspase-3. Simvastatin (sim) decreases the activity of caspase-3 at Days 1 and 3 after treatment compared with the saline-treated group.](image)

...and subsequent neurological functional deficits in the animals. These areas are potentially salvageable. Rescuing the damaged neurons in these areas promotes functional outcome after brain injury. Therefore, neuronal survival in these areas is an important target to evaluate the degree of damaged brain tissue and the effect of therapeutic intervention. In our current study, significant functional improvements in the simvastatin-treated group were detected by the mNSS at Days 7 and 14 after treatment when compared with the saline-treated control group. Significant functional improvement occurring at Day 7 after treatment suggests that simvastatin may rescue damaged neurons. This hypothesis was supported by the decreased numbers of TUNEL-positive cells in the boundary zone and the hippocampus in the ipsilateral hemisphere after simvastatin treatment (Fig. 3).

To address the potential mechanisms by which simvastatin exerts its protective effects, we analyzed the caspase-3 activity in tissue samples obtained from different time points. Caspases, the ICE/CED-3 family of proteases, play a central role in the early stages of apoptosis. Of the identified caspases, caspase-3 is activated during apoptosis in many types of central nervous system cells, and its activation appears to be an important event in apoptosis. Previous studies have shown increased apoptosis and caspase-3 activity in a rat TBI model. Simvastatin significantly decreased caspase-3 activities on Days 1 and 3 after treatment, suggesting that simvastatin inhibits the caspase-3 activation in the early phase. These results are consistent with previous data on the effect of simvastatin on caspase-3 activity in a hypoxia-ischemia model in the newborn rat.

Given that simvastatin may prevent apoptotic cell death through inhibition of caspase-3 activation, we further explored its underlying signaling pathway. The effect of simvastatin on phosphorylation of Akt has been reported in endothelial cells in vitro. Recent studies have suggested that simvastatin induces the phosphorylation of Akt, eNOS, IκB, and FOXO1. Western blot analysis shows that simvastatin induces the phosphorylation of Akt, eNOS, IκB, and FOXO1.

Because Akt phosphorylates FOXOs as downstream targets in cell survival signaling, we examined changes in FOXO1 phosphorylation as well as Akt phosphorylation after TBI. The FOXOs regulate the transcription of the cell cycle inhibitor p27 and the Bcl-2–like protein Bim, both of which are strong proapoptotic signaling proteins. Akt phosphorylates and inactivates FOXO1 which prevents its translocation to the nucleus, thereby inhibiting apoptotic signaling. The FOXO1 in neurons normally stays in the cytoplasm because of Akt-dependent phosphorylation. Once Akt activity is reduced by brain injury conditions, FOXO1 is dephosphorylated by undefined protein phosphatases. The dephosphorylation contributes to their importation into the nucleus through nuclear localization signals. The DNA-binding domain is required for activation of the promoters of Fas-ligand.
and Bim genes. Expression of the Fas-ligand gene induces caspase activation, which eventually leads to apoptosis. Simvastatin significantly increases phosphorylation of FOXO1 at an early phase in an Akt-dependent manner, and thereby, likely rescues neurons from apoptosis through phosphorylation and inactivation of FOXO1.

Nuclear factor–κB is widely known for its ubiquitous roles in the immune response as well as in control of cell
division and apoptosis. Functional NF-κB complexes are present in essentially all cell types in the nervous system, including neurons. In its inactive form, NF-κB is present in cytosol as a 3-subunit complex, with the prototypical components being p65 and p50 and IkB (inhibitory subunit). Nuclear factor–κB is activated by signals that activate IkB kinase, resulting in phosphorylation of IkB; this targets IkB for degradation in the proteosome and frees the p65-p50 dimer, which then translocates to the nucleus and binds to consensus κB sequences in the promoter region of κB-responsive genes. The expression of several different genes that promote neuron survival is induced by NF-κB, including those encoding manganese superoxide dismutase (Mn-SOD), inhibitor-of-apoptosis proteins, and Bcl-2. These prosurvival proteins prevent the injured cells from undergoing the apoptotic process. Levels of NF-κB activity are increased in the cerebral cortex within hours of TBI in rats, after which they remain elevated for ≥24 hours. Our data show that simvastatin elevates phosphorylation of IkB, which has been proven to promote the activation of NF-κB. The elevated phosphorylation of IkB lasts for 7 days after simvastatin treatment, inducing a prolonged activation of NF-κB, which may mediate the antiapoptotic effect of simvastatin.

The Akt-dependent phosphorylation leads to the post-translational activation of the eNOS via phosphorylation of the amino acid Ser177, which has been described as an important antiapoptotic signaling pathway in endothelial cells. Uregulated expression of eNOS leads to an increase in cerebral blood flow, smaller cerebral infarcts, and decreased neurological deficits in stroke. Recent studies suggest that simvastatin can rapidly activate the protein kinase Akt/PKB in endothelial cells. Accordingly, simvastatin enhances phosphorylation of the endogenous Akt substrate eNOS and inhibits apoptosis in vitro in an Akt-dependent manner. In the TBI model, we find a similar pattern of elevated phosphorylation of eNOS after treatment with simvastatin.

Conclusions

Based on our findings, we propose that treatment with simvastatin increases phosphorylation of Akt in the neuronal cells after TBI, followed by phosphorylation of its downstream targets FOXO, IkB, and eNOS, which eventually leads to antiapoptotic effects partially through suppressing caspase-3 activity.

Our data suggest that simvastatin suppresses apoptosis of neuronal cells and enhances the sensory-motor functional recovery post-TBI. The Akt/FOXO/IkB/caspase-3 pathway may play a vital role in the neuroprotective effect of simvastatin treatment after TBI.

Disclosure

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References