The PI3K/Akt Pathway Mediates the Neuroprotective Effect of Atorvastatin in Extending Thrombolytic Therapy After Embolic Stroke in the Rat

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Objective—We tested the hypothesis that the phosphatidylinositol-3 kinase (PI3K)/Akt pathway mediates the neuroprotective effect of combination therapy of atorvastatin and tissue-type plasminogen activator (tPA) in rats after stroke.

Methods and Results—Combination of atorvastatin (20 mg/kg) and tPA (10 mg/kg) significantly reduced ischemic lesion volume, whereas monotherapy with atorvastatin and tPA did not reduce the lesion volume, when the treatments were initiated 4 hours after embolic middle cerebral artery occlusion (MCAo). Western blot analysis revealed that treatment with atorvastatin alone and in combination treatment with tPA significantly increased Akt phosphorylation compared with treatment with saline and tPA alone. Inhibition of the PI3K/Akt pathway with wortmannin completely abolished the reduction of lesion volume afforded by combination of atorvastatin and tPA. Real-time RT-PCR analysis of cerebral endothelial cells isolated by laser-capture microdissection from the ischemic boundary region showed that MCAo upregulated early growth response 1 (Egr-1) and vascular endothelial growth factor (VEGF) mRNA levels and tPA monotherapy further increased Egr-1 and VEGF mRNA levels. However, combination of atorvastatin and tPA significantly suppressed Egr-1 and VEGF mRNA levels in cerebral endothelial cells.

Conclusions—Activation of Akt and downregulation of cerebral endothelial Egr-1 and VEGF gene expression by atorvastatin contribute to the neuroprotective effect of combination treatment with atorvastatin and tPA. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: ●●●

Statins are a class of 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase inhibitors, which are widely prescribed to reduce cholesterol levels in hyperlipidemic patients.1 Evidence from experimental stroke and stroke patients demonstrates that the prophylactic effects of statins on stroke extend beyond the scope of their cholesterol lowering actions.2 In addition to their prophylactic benefits, statins have been used for the treatment of acute stroke.3–6 Treatment of experimental stroke with short-term high-dose atorvastatin in combination with tissue-type plasminogen activator (tPA) extends the therapeutic window for tPA to at least 4 hours after stroke onset.4 The neuroprotective effects of combination treatment with atorvastatin and tPA are associated with the enhancement of cerebral vascular patency and integrity, whereas tPA alone exacerbates cerebral vascular disruption, indicating that atorvastatin provides protection from ischemic brain damage, at least partly, by cerebrovascular mechanisms.4,7

The prophylactic benefit of statin treatment of stroke is mainly mediated by eNOS, which augments cerebral blood flow (CBF).8–10 However, recent reports demonstrate that in addition to their effects on eNOS, statins exert their neuroprotective effect on stroke through many other actions.4,6,11,12 Studies in postnatal rats subjected to hypoxia-ischemia showed that treatment with simvastatin reduced brain injury and improved functional recovery, which was associated with a reduction in cytokine expression, but not changes in eNOS expression.13 In a mouse model of focal cerebral ischemia, rosuvastatin reduced tPA-aggravated brain damage by a mechanism independent of eNOS.6 We previously demonstrated that administration of atorvastatin after stroke extended the therapeutic window of tPA without an increase of eNOS levels, and the neuroprotective effects of combination of tPA and atorvastatin were not abolished in eNOS−/− mice.4 Thus, there are other potential mechanisms that underlie the beneficial effect of statins on the acute treatment of stroke. The phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway plays a central role in regulating cell growth, proliferation, and survival under physiological and pathophysiologic conditions.13,14 The activation of the PI3K/Akt pathway negatively modulates genes that promote thrombo-
genicity, vascular permeability, and inflammation, and thereby protects vascular function.15–18 Statins induce Akt translocation to the plasma membrane of endothelial cells, which promotes the activating phosphorylation of Akt at serine 473.19 Activation of the PI3K/Akt pathway by statins significantly reduces infarct size in cardiac ischemia.20 Accordingly, using a rat model of embolic middle cerebral artery occlusion (MCAo), we tested the hypothesis that the PI3K/Akt pathway mediates the extension of the therapeutic window of tPA by atorvastatin.

Materials and Methods
All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal Model
Male Wistar rats weighing 350 to 450 g were subjected to embolic middle cerebral artery occlusion (MCAo).21 Briefly, the animals were anesthetized with 4% isoflurane during induction and then maintained with 2% isoflurane in a mixture of 30% O2 and 70% N2O. Body temperature was monitored and maintained at 37°C using a feedback-regulated water heating system. Under the operating microscope (Carl Zeiss), the right common carotid artery (CCA), the right external carotid artery (ECA), and the internal carotid artery (ICA) were isolated via a midline incision. A modified PE-50 catheter with a 0.3 mm outer diameter was gently advanced from the ECA into the lumen of the ICA until the tip of the catheter reach the origin of the MCA (≈15 to 16 mm). Through the catheter, a single clot (∼0.8 µL) along with 2 to 3 µL of 0.9% saline was then gently injected. The catheter was withdrawn immediately after injection, and the right ECA was ligated.

Experimental Protocols
After MCAo, animals were randomly assigned to 5 groups. To clarify whether the PI3K/Akt pathway contributes to the neuroprotective effects of combination of atorvastatin and tPA, wortmannin, a selective PI3-K inhibitor, was given intravenously at a dose of 15 µg/kg at 3/5 hour after embolic MCA, followed by atorvastatin and tPA at 4 hours after stroke onset (n = 10).

Western Blot Analysis
Rats treated with saline (n = 15), tPA (n = 15), atorvastatin (n = 15), combination of atorvastatin and tPA (n = 15), and wortmannin (n = 8) served as control groups.

Measurements of Infarct Volume
Seven days after MCAo, ischemic lesion volume was measured on 7 hematoxylin and eosin (H&E)-stained coronal sections, as previously described.21 Data are presented as the percentage of the contralateral hemisphere.

Results

Figure 1. Brain Akt levels. A, Western blot analysis of phosphorylated and total Akt from representative normal rats, and rats treated with saline, atorvastatin, tPA, wortmannin, and combination of atorvastatin and tPA 4 hours after MCA occlusion. Bar graph (B) shows quantitative data of phosphorylated Akt from each group. Values are mean±SE. *P<0.05 as compared with nonischemic rats, **P<0.05 as compared with the saline treated group.

With an antibody against von Willebrand factor (vWF) (1:50 dilution) for 3 min and exposed to CY3 conjugated F(ab')2, secondary antibody (DAKO, 1:50 dilution). Using a PixCell IIe (LCM) Instrument (Arcturus Engineering), vWF immunoreactive cerebral endothelial cells (approximately 500 cells per rat) were isolated from the ischemic boundary region. Total RNA was isolated using the RNeasy Micro Kit (Qiagen Inc). Quantitative RT-PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems) using 3-stage program parameters provided by the manufacturer. Each sample was assayed in triplicate and the Taqman PCR CT values obtained from three independent experiments were used for analysis of relative gene expression using the 2−△△Ct method.22 The TaqMan primer/hybridization probes for genes of VEGF and Egr-1, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from ABI.

Statistics
Data are presented as the mean±SE. One-way ANOVA was used to compare multiple-group values (ie, measurements of lesion volume, phospho-Akt, total-Akt, Egr-1, and VEGF levels). If the main effect of treatment group was significant at P<0.05, then all pair-wise comparisons between treatment groups were tested. Adjustments for multiple comparisons were made using Hochberg’s method.

Results
Atorvastatin alone and in combination with tPA activates phosphorylation of Akt: Western blot analysis revealed that stroke significantly increased phosphorylation of Akt compared with non-ischemic rats (Figure 1), which is consistent with published studies.23 Treatment with atorvastatin alone and in combination with tPA significantly increased Akt phosphorylation compared with saline and tPA treated rats at...
6 hours after MCAo (Figure 1). However, treatment with tPA alone resulted in a trend toward reduction of Akt phosphorylation compared with saline treated rats after stroke (Figure 1). No differences of total Akt were detected among groups (Figure 1).

Wortmannin abrogates the neuroprotection induced by combination of atorvastatin and tPA: Combination treatment with atorvastatin and tPA significantly (P<0.05) reduced ischemic lesion volume compared with saline-treated and tPA-treated rats 7 days after stroke (Figure 2). To examine whether activated Akt has a biological effect on stroke outcome, wortmannin was administered. Treatment with wortmannin alone 4 hours after stroke did not significantly increase ischemic lesion volume (36.9±3.1%) compared with that in rats treated with saline (37.5±3.3%). However, administration of wortmannin completely abolished the infarct size reduction afforded by combination treatment of stroke with atorvastatin and tPA (Figure 2). Wortmannin also suppressed Akt activated by the combination therapy (Figure 1).

**Effect of Combination of Atorvastatin and tPA on Egr-1**

Egr-1 triggers inflammation, coagulation, and vascular permeability underlying ischemic stress. Using Taqman probes, Egr-1 mRNA levels were measured in the cerebral endothelial cells obtained with the LCM technique from brain coronal sections 30 hours after stroke onset. Real time RT-PCR analysis revealed that stroke induced approximately a 10-fold increase of Egr-1 levels compared with levels in endothelial cells extracted from nonstroke rats (Figure 3). Monotherapy with tPA further increased Egr-1 levels to 20-fold compared with nonstroke rats and resulted in a trend toward increase of Egr-1 levels compared with saline treated rats after stroke (Figure 3A). However, treatment with atorvastatin significantly reduced Egr-1 levels compared with that in rats treated with saline or tPA alone after stroke. Moreover, combination treatment with atorvastatin and tPA reversed the stroke and tPA induced upregulation of Egr-1 in rats after stroke (Figure 3A).

**Effect of Combination of Atorvastatin and tPA on VEGF**

VEGF increases microvascular permeability and promotes angiogenesis. To examine the effects of the combination treatment on cerebral microvascular VEGF expression, real time RT-PCR analysis of VEGF mRNA was performed in the cerebral endothelial cells. Stroke induced a significant increase of VEGF mRNA levels compared with that in normal rats (Figure 3B). Ischemic rats treated with tPA alone exhibited an even greater increase of VEGF levels compared with nonstroke rats (Figure 3B). However, the combination therapy significantly reduced VEGF levels compared with that in rats treated with tPA alone (Figure 3B).

**Discussion**

The present study demonstrates that combination treatment with short-term high-dose atorvastatin and tPA significantly increased phosphorylation of Akt, and inhibition of the PI3K/Akt pathway with wortmannin abolished the neuroprotective effect of the combination treatment. In addition, stroke rats treated with combination of atorvastatin and tPA significantly reduced Egr-1 and VEGF mRNA levels in cerebral endothelial cells compared with rats treated with tPA monotherapy. These data suggest that the PI3K/Akt signaling pathway activated by atorvastatin mediates the neuroprotective effect of thrombolysis on ischemic stroke. Activated Akt may downregulate endothelial Egr-1 and VEGF gene expression, and thereby reduces ischemic brain damage. Additional experiments to investigate how Akt mediates the regulation of Egr-1 and VEGF by a statin are warranted.

We recently documented that combination short-term treatment with high-dose atorvastatin and tPA effectively reduced ischemic brain damage in rats after embolic stroke. The neuroprotective effect of the combination

![Figure 2. Ischemic lesion volume. Bar graph shows the percentage of lesion volume assessed 7 days after MCA occlusion. Values are mean±SE. *P<0.05 as compared with the saline-treated group.](http://atvb.ahajournals.org/)

![Figure 3. Cerebral endothelial Egr-1 and VEGF gene expression. Quantitative data of real time RT-PCR of Egr-1 (A) and VEGF (B) mRNA levels in cerebral endothelial cells isolated from normal and ischemic rats treated with saline, atorvastatin, tPA, and combination of atorvastatin and tPA. Values are mean±SE. *P<0.05 as compared with nonischemic rats. **P<0.05 as compared with the saline treated group.](http://atvb.ahajournals.org/)
treatment was not abolished in eNOS knockout mice. Others have shown that when rosuvastatin and tPA were coadministered immediately after transient (90 minutes) MCA occlusion in mice, infarct volume was significantly reduced compared with the volume in tPA alone group independent of eNOS. These data indicate that in addition to eNOS, other mechanisms contribute to the neuroprotective effect of statins. Statins activate phosphorylation of Akt, which has been demonstrated extensively in vitro and in experimental models of myocardial ischemia. However, the effects of high-dose short-term atorvastatin on phosphorylation of Akt after stroke have not been investigated. The present data demonstrated for the first time that treatment with short-term high-dose atorvastatin in combination with tPA rapidly increases phosphorylated Akt levels which is associated with reduction of ischemic brain damage. In addition, blocking PI3K/Akt with wortmannin abolished activation of Akt and the neuroprotective effect resulted from the combination treatment. Thus, our data indicate that the PI3K/Akt pathway mediates the neuroprotective effect of atorvastatin in extending tPA thrombolytic therapy. However, the present study does not rule out the neuroprotective effect of eNOS, as the PI3/Akt signaling pathway activates eNOS. It is interesting to note that treatment with wortmannin alone 4 hours after stroke significantly reduced Akt phosphorylation but did not increase ischemic lesion volume (37%) compared with rats treated with saline (38%). In our model of embolic MCAo, the infarct volume is approximately 35% of the contralateral hemisphere and nearly encompasses the entire territory supplied by the MCA. Therefore, blockade of Akt activation may not further enlarge ischemic lesion volume. The present findings are in accordance with previous results in experimental myocardial ischemia, which demonstrated that treatment with simvastatin acutely activated Akt, whereas blockage of the PI3/Akt pathway attenuated the beneficial effects of simvastatin on myocardial reperfusion injury. The beneficial effect of statins on thrombolysis with tPA has been recently reported in stroke patients, which shows that pretreatment with statins improves stroke outcome in patients who underwent thrombolytic therapy with tPA. Studies on tPA knockout mice demonstrate that the atorvastatin reduces infarct volume in mice subjected to filament but not to embolic MCAo, suggesting that atorvastatin enhances thrombolysis via upregulation of endogenous tPA. Interestingly, our data showed that although it activated Akt, atorvastatin monotherapy initiated 4 hours after stroke did not have the neuroprotective effect. These data are consistent with our previous findings that treatment with atorvastatin alone 4 hours after stroke failed to reduce progressive cerebral microcirculatory impairment, whereas combination of atorvastatin and tPA enhanced cerebral vascular patency. Collectively, these data suggest that without exogenous tPA, activation of Akt by atorvastatin is not sufficient to improve the microcirculation and consequently to salvage ischemic cell damage. Additional studies on spatial and temporal profile of Akt activated by atorvastatin are warranted.

The PI3K/Akt signaling pathway plays a key role in many cellular processes, including cell survival, coagulation, and inflammation responses. Egr-1, downstream of Akt, is critical for regulating vascular thrombotic, inflammatory events and BBB permeability. Indeed, using the inducible form of Akt to activate Akt, others show that activation of Akt significantly decreases Egr-1 expression. In the vasculature, Egr-1 is rapidly upregulated under ischemic stress. Upregulation of Egr-1 induces expression of multiple target genes that regulate inflammatory and coagulant pathways. Egr-1-deficient mice exhibit suppression of tissue factor, plasminogen activator inhibitor (PAI)-1, intercellular adhesion molecule-1 (ICAM-1), and VEGF gene expression. The present study shows that MCAo upregulated Egr-1 expression in endothelial cells, which was further upregulated by monotherapy of tPA. However, atorvastatin blocked tPA-induced upregulation of Egr-1. We previously demonstrated the multi-targeted effects of atorvastatin on cerebral vascular patency and integrity to enhance tPA thrombolytic therapy of stroke. In parallel with our findings, others demonstrate that in apolipoprotein E-deficient mice, treatment with simvastatin reduces Egr-1 expression and subsequently reduces the expression of multiple genes that promote thrombosis formation and inflammatory responses. Egr-1 acts as a master switch to trigger expression of pivotal regulators of coagulation, vascular permeability, and inflammation. Collectively, these data suggest that downregulation of Egr-1 by activated Akt through atorvastatin likely contributes to the multi-targeted effects of atorvastatin. However, it is worth noting that as a pleiotropic factor, other pathways activated by statin treatment may regulate Egr-1 expression. For example, statins block mitogen-activated protein kinase (MEK1)/extracellular signal regulated kinase (ERK) (Erk) signaling pathway, which has been shown to regulate Egr-1 expression. Furthermore, data of phosphorylated Akt were obtained from brain tissue, whereas results of Egr-1 and VEGF expression were from cerebral endothelial cells. Additional studies on cerebral endothelial cells are warranted to define the pathways involved in statin mediated Egr-1 inhibition.

The role of VEGF in ischemic brain is multifaceted depending on temporal and spatial profiles of VEGF. During the acute stage of ischemic injury, upregulation of VEGF in cerebral vessels increases BBB permeability resulting in exacerbation of ischemic cell damage. However, by preventing BBB leakage, acute intraventricular infusion of VEGF protects ischemic neuronal damage. High doses of statins downregulate VEGF expression. Consistent with the published studies, the present data show that atorvastatin in combination with tPA reduced VEGF expression in the cerebral endothelial cells and had a neuroprotective effect, suggesting that the acute reduction of endothelial VEGF likely contributes to beneficial effects of combination of atorvastatin and tPA. However, whether downregulation of VEGF observed in the present study resulted from activation of Akt or from a direct effect of atorvastatin remains unknown. In addition, VEGF...
triggers angiogenesis at a later stage of stroke.\textsuperscript{38} Further studies on the precise timing and spatial localization of VEGF may shed light on the effect by which the acute high dose of atorvastatin mediate VEGF expression and angiogenesis in ischemic brain.

In conclusion, combination of short-term high-dose atorvastatin and tPA exerts a neuroprotective effect via PI3K/Akt pathway-dependent mechanisms. The activation of the PI3K/Akt pathway by atorvastatin may suppress upregulation of Egr-1, which enhances thrombolytic therapy of tPA of stroke.

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Disclosures

None.

References


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