eNOS Mediates TO90317 Treatment-Induced Angiogenesis and Functional Outcome After Stroke in Mice

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Background and Purpose—TO901317, a synthetic liver X receptor agonist, elevates high-density lipoprotein cholesterol (HDL-C) in mice. We tested the hypothesis that TO901317 treatment of stroke promotes angiogenesis and vascular maturation and improves functional outcome after stroke by increasing endothelial nitric oxide synthase (eNOS) phosphorylation.

Methods—C57BL/6J mice were subjected to middle cerebral artery occlusion and were treated with or without TO901317 (30 mg/kg) starting 24 hours after middle cerebral artery occlusion and daily for 14 days.

Results—TO901317 significantly increased serum HDL-C level, promoted angiogenesis and vascular stabilization in the ischemic brain, and improved functional outcome after stroke. The increased HDL-C level significantly correlated with functional recovery after stroke. TO901317 also increased eNOS phosphorylation in the ischemic brain. Mechanisms underlying the TO901317-induced angiogenesis were investigated using eNOS knockout (eNOS−/−) mice. TO901317 treatment of eNOS−/− mice significantly increased HDL-C level but failed to increase angiogenesis and functional outcome after stroke. In vitro studies demonstrated that TO901317 and HDL-C significantly increased capillary tube formation and promoted eNOS phosphorylation activity in cultured mouse brain endothelial cells compared with nontreatment controls. However, TO901317 and high-density lipoprotein treatment-induced capillary tube formation were absent in eNOS-deficient mouse brain endothelial cell.

Conclusions—These data indicate that TO901317 treatment increases serum HDL-C level, which promotes angiogenesis through eNOS and leads to improvement of functional outcome after stroke. (Stroke. 2009;40:2532-2538.)

Key Words: angiogenesis ■ eNOS ■ HDL-C ■ stroke ■ TO901317

High-density lipoprotein cholesterol (HDL-C) is a heterogeneous group of lipoproteins exhibiting a variety of properties, eg, decrease in platelet aggregation and inhibition of endothelial cell (EC) apoptosis.1 HDL-C enhances vasorelaxation and promotes EC migration and re-endothelialization by increasing endothelial nitric oxide synthase (eNOS) expression and eNOS phosphorylation (p-eNOS).2-5 Reconstituted high-density lipoprotein treatment of acute myocardial infarction rats improves cardiac function after myocardial infarction in rats.6 Niacin treatment of stroke rats increases HDL-C level, upregulates p-eNOS and angiogenesis, and improves functional outcome.7 Higher levels of HDL-C are associated with better functional performance in the elderly.8 Low HDL-C predicts poor cognitive function and worse disability after stroke.9 Clinical studies have shown that statins significantly lower cholesterol and are not very effective at increasing high-density lipoprotein levels. Thus, agents that increase the HDL-C level may be attractive targets as restorative treatments for stroke.

TO901317, a potent nonsteroidal synthetic liver X receptor (LXR) agonist, elevates HDL-C and phospholipid and generates enlarged HDL-C particles enriched in cholesterol.10 LXR agonists control the expression of several genes important for cholesterol homeostasis in the brain.11 LXR knockout (LXR−/−) mice exhibit enlarged brain blood vessels with weak staining of α-smooth muscle actin (α-SMA) and excessive lipid accumulation around the abnormal vessels, which lose their contractile ability and are susceptible to rupture.12 Activation of LXRs promotes neuroprotection and decreases expression of proinflammatory genes and reduces nuclear factor-κB transcriptional activity in experimental stroke.13,14 TO901317 suppresses the vascular inflammatory status and lowers lesional macrophage accumulation.15 However, there are no studies that evaluate whether TO901317 treatment regulates angiogenesis and promotes neurorestoration after stroke.

In this study, we tested a novel hypothesis, that increasing HDL-C by TO901317 treatment promotes angiogenesis in the ischemic brain as well as improves functional outcome after stroke in mice. In addition, the mechanisms and molecular signaling pathway of TO901317-induced angiogenesis were investigated.
Vascular Density Measurement
Five slides from the vWF-immunostained coronal section with each slide containing 8 fields of view within the IBZ were digitized. The total vascular density in the IBZ was measured in each section as previously described.24 The total number of vWF-positive vessels per square millimeter area is presented.

α-SMA-Positive Coated Vessel Density Measurement
The density of α-SMA-stained vessels was analyzed with regard to small and large vessels (≥10 μm diameter) in the IBZ. Five sections and 8 brain regions within each section were acquired and numbers of α-SMA-immunoreactive vessels were counted. The total number of α-SMA-positive coated vessels per square millimeter area is presented.

Mouse Brain Endothelial Cell Culture
Mouse brain endothelial cell (MBECs; ATCC, CRL-2299) culture was used. MBECs were treated with: (1) nontreatment control; and (2) TO901317 0.1 μmol/L and 1 μmol/L, respectively (n=3/group). Cells were treated for 12 hours before harvesting for real-time polymerase chain reaction and Western blot assays.

Real-Time Polymerase Chain Reaction
MBECs were harvested and total RNA was isolated following a standard protocol.25 Quantitative polymerase chain reaction was performed on an ABI 7000 polymerase chain reaction instrument (Applied Biosystems, Foster City, Calif) using 3-stage program parameters provided by the manufacturer. Each sample was tested in triplicate and analysis of relative gene expression data using the 2−ΔΔCT method was done. The following primers for real-time polymerase chain reaction were designed using Primer Express software (ABI): eNOS—forward: TTG AAA ATG AGA CTT GTT CAA TGC; reverse: TGC AGA GTC GAT GGT TAC AAG GAG. GAPDH—forward: AGA ACA TCA TCC CTG CAT CC; reverse: CAC ATT GGG GGT AGG AAC CT.

Western Blot
Equal amounts of cell or brain tissue lysate were subjected to Western blot analysis as previously described.24 Specific proteins were visualized using a SuperSignal West Pico chemiluminescence kit (Pierce). The primary antibodies used were: anti-β-actin (1:2000; Santa Cruz Biotechnology, Santa Cruz, Calif), anti-eNOS (1:1000; Cell Signaling Technology), and antiphospho-eNOS (Ser1177; 1:1000; Cell Signaling Technology).

eNOS Deficit Mice Brain MBEC Culture
eNOS−/− mouse brains were collected. The cortical brain tissue was isolated and digested in collagenase/Dispase and the microvessels separated by centrifugation in a Percoll (Sigma) gradient. Microvessels were seeded in flasks coated with rat-tail collagen and the medium was changed every 2 to 3 days. eNOS−/− MBECs were used for tube formation assay.

Capillary-Like Tube Formation Assay
MBECs (2×10^4 cells) were incubated in (n=6/group): (1) regular cell culture medium (DMEM) for control; (2) TO9013117 0.1 μmol/L; 1 μmol/L, and 10 μmol/L; (3) HDL-C (80 μmol/L and 160 μmol/L); (4) eNOS−/− MBECs alone; (5) eNOS−/− MBECs treated with TO901317 (1 μmol/L); and (6) eNOS−/− MBECs treated with high-density lipoprotein (80 μg/mL) for 5 hours. All assays were performed in triplicate and total length of the tube-like formation was quantitated.26

Statistical Analysis
Independent samples t test was used for testing functional outcome, HDL-C, p-eNOS expression, and number of vWF- or α-SMA-positive vessels between the 2 groups. One-way analysis of variance and least significant difference analysis after post hoc testing were performed to assess eNOS mRNA, p-eNOS expression, and tube formation in vitro. Two-way analysis of variance was performed for measurement of BrdU, occludin, and vWF-α-SMA-positive vessels in the ischemic brain. If an overall treatment group effect was detected at P<0.05, Tukey test after post hoc testing was used for
multiple comparison. Pearson partial correlation after bivariate correlation was used to analyze the correlation effect. All data are presented as mean ±SE.

Results

TO901317 Treatment Improves Neurological Outcome and Increases Serum HDL-C Level
Figure 1A shows that WT mice treated with TO901317 significantly improved functional recovery in foot-fault test compared with control MCAO animals (P < 0.05). No significant differences of infarct volumes in the TO901317-treated group (14.9% ± 4.2%) were detected compared with the MCAO control (18.8% ± 2.3%). Serum of HDL-C significantly increased at 14 days in the TO901317-treated group compared with the nontreatment control MCAO (Figure 1B; P < 0.05). Correlation coefficient analysis shows a strong negative correlation between foot-fault and HDL-C level at 14 days after treatment (r = –0.78, P < 0.05). These data indicate that TO901317 treatment of stroke increases HDL-C and the increased HDL-C correlates with functional outcome after stroke.

TO901317 Treatment of Stroke Increases Angiogenesis and Vascular Maturation in the Ischemic Brain
Figure 2A–B show that BrdU-positive ECs (P = 0.001, F = 12.653, Figure 2A) and vascular density (P = 0.004, F = 4.903, Figure 2B) were significantly increased in the TO901317 treatment groups compared with control animals. Figure 2C–D shows that treatment with TO901317 significantly (P < 0.05) increased occludin expression (P = 0.018, F = 4.664, Figure 2D) and α-SMA-positive vessel density (P = 0.042, F = 3.716, Figure 2C) in the IBZ area compared with the control MCAO mice.

To test the mechanism that underlies TO901317-induced angiogenesis, eNOS and p-eNOS expression were measured in the ischemic brain. Figure 2E shows that TO901317 treatment of stroke significantly increases p-eNOS activity in the ischemic brain (P < 0.05).

eNOS Is Required for TO901317-Induced Functional Outcome After Stroke
TO901317 treatment in eNOS+/− mice increases HDL-C level (74.4 ± 5.1 mg/dL) compared with eNOS+/− MCAO controls (48.4 ± 4.2 mg/dL, P < 0.05) but fails to improve functional outcome after stroke (Figure 1C). No significant differences of infarct volumes in the TO901317-treated eNOS+/− mice (18.6% ± 6.5%) were detected compared with eNOS+/− MCAO controls (19.3% ± 2.0%). In addition, treatment with TO901317 in eNOS−/− mice did not significantly increase vWF-positive vessel density (Figure 2F) and α-SMA-positive vessel density (Figure 2G) in the IBZ compared with the eNOS−/− MCAO controls.

TO901317 and High-Density Lipoprotein Increase MBEC p-eNOS
Figure 3A–C shows that TO901317 and high-density lipoprotein treatment do not regulate eNOS gene and protein expression but significantly promote p-eNOS activity compared with nontreatment controls (P < 0.05).

TO901317 and HDL-C Induce Angiogenesis In Vitro
Figure 4A–D shows that TO901317 and HDL-C dose dependently increased capillary tube formation compared with control DMEM medium.

eNOS Mediates TO901317 and High-Density Lipoprotein-Induced Tube Formation
Figure 4E shows that capillary tube formation significantly decreased in eNOS−/− MBECs compared with WT MBECs. TO901317 (1 μmol/L) and high-density lipoprotein (80 μg/mL) do not significantly increase capillary tube formation in eNOS−/− MBECs compared with eNOS−/− MBEC controls (P > 0.05).

Discussion

TO901317 Increases HDL-C and Improves Functional Outcome After Stroke
TO901317 is a potent LXR agonist. LXRs activate reverse cholesterol transport, including the ATP binding cassette transporter A1, and raise HDL-C levels.27,28 Intravenous injection of reconstituted HDL (rHDL) significantly augments blood flow recovery and increases capillary density in the ischemic leg.29 Patients with stroke exposed to power-frequency electromagnetic fields, which increase HDL-C, show a statistically significant better prognosis compared with the control group.30 In this study, we found that TO901317 treatment significantly increases serum HDL-C and promotes functional outcome after stroke. Increased HDL-C correlated with functional outcome after stroke.
Therefore, increasing HDL-C by TO901317 treatment may contribute to functional outcome after stroke.

TO901317 Increases Angiogenesis and Vascular Maturation After Stroke

Angiogenesis involves the sprouting, branching, splitting, and differential growth of vessels in the primary plexus to form the mature vascular system. During angiogenic vascular remodeling, supporting cells such as pericytes and smooth muscle cells are recruited to the vessels to provide structural support and stability for the vascular walls. TO901317 treatment of stroke induces angiogenesis identified by increasing EC proliferation and vascular density and also promotes smooth muscle cell adhesion to vessels and increases tight junction protein occludin expression in vessels in the ischemic brain. These data suggest that TO901317 treatment of stroke not only induces angiogenesis, but also promotes vascular maturation. However, recovery of neurological function after stroke is mediated by many coupled events, including vascular remodeling, neurogenesis, and synaptogenesis. We do not exclude the possibility that other restorative events, in addition to angiogenesis, contribute to recovery of function. Whether TO901317 regulates neurogenesis and synaptogenesis warrants further investigation.

eNOS Mediates TO901317-Induced Angiogenesis After Stroke

High-density lipoprotein stimulates eNOS activation. The antiatherogenic role of high-density lipoprotein is also related to the increased activity of eNOS. HDL-C promotes EC migration and re-endothelialization mediated by activation of eNOS.

**Figure 3.** TO901317 regulates eNOS expression in cultured MBECs. A, eNOS gene expression; (B–C) Western blot (B) and quantitative data (C). N=3/group.

**Figure 4.** TO901317 and high-density lipoprotein regulate capillary-like tube formation. Inhibition of eNOS attenuates TO901317 and high-density lipoprotein-induced tube formation. A–D, Capillary-like tube formation in WT MBEC control (A), TO901317 (1 μM; B), and high-density lipoprotein (80 μg/mL; C) and quantitative data (D). E, Quantitative data of capillary-like tube formation in eNOS−/− MBECs. N=6/group. Scale bar in A = 0.5 mm.
eNOS.4 HDL-C promotes eNOS activity by maintaining the lipid environment in caveolae where eNOS is colocalized with partner signaling molecules.36 Enhanced p-eNOS induces a broad range of effects, including the promotion of angiogenesis and mural cell recruitment to immature angiogenic sprouts.37 EC-derived nitric oxide induces mural cell recruitment as well as subsequent morphogenesis and stabilization of angiogenic vessels.38 Our data show that TO901317 treatment of stroke increases HDL-C level and promotes phosphorylation of eNOS in the ischemic brain. TO901317 and HDL-C treatment of MBEcs significantly increase p-eNOS activity as well as promote angiogenesis compared with controls. We tested the profile of TO901317 dose-dependent regulation of tube formation in vitro. The reason why high-dose TO901317 (10 μmol/L) reduces tube formation warrants further investigation. To elucidate the contribution of eNOS to TO901317-mediated angiogenesis, we used eNOS−/− mice. We found that TO901317 treatment of stroke in eNOS−/− mice increases HDL-C level but failed to improve functional outcome after stroke as well as regulate angiogenesis compared with nontreatment eNOS−/− mice. These data indicate that eNOS plays a critical role in TO901317-induced angiogenesis and functional outcome after stroke.

Summary
We demonstrate that treatment of experimental stroke with TO901317 24 hours after stroke significantly increases HDL-C levels, promotes angiogenesis, and improves functional outcome after stroke. eNOS appears to mediate TO901317-induced angiogenesis.

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Disclosures
None.

References


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