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

Jieli Chen, MD; Xu Cui, PhD; Alex Zacharek, MS; Cynthia Roberts, BS; Michael Chopp, PhD

**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 LXRs 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.
**Materials and Methods**

**Animal Middle Cerebral Artery Occlusion Model and Experimental Groups**

Adult male wild-type (WT) C57BL/6J and C57BL/6J eNOS knockout (eNOS−/−) mice (age 2 to 3 months, weight 24 to 28 g) were purchased from Charles River (Wilmington, Mass). Right temporal (2-hour) middle cerebral artery occlusion (MCAO) was induced using the filament model as previously described. Sham-operated mice underwent the same surgical procedure without suture insertion. MCAO and sham-operated WT mice were gavaged starting 24 hours after surgery with: (1) saline for control; or (2) TO901317 (30 mg/kg; Cayman Chemical). Our choice of dose was guided by a previous study. To test whether eNOS mediates TO901317-induced functional outcome after stroke, eNOS−/− mice were used. eNOS−/− mice were subjected to 2-hour MCAO and treated with or without TO901317 (30 mg/kg) starting 24 hours after MCAO daily for 14 days. All mice received daily intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg; Sigma, St Louis, Mo), a thymidine analog, which labels newly synthesized DNA, starting at 24 hours after MCAO daily for identification of cell proliferation. Mice (n=8/group) were euthanized 14 days after MCAO for immunostaining. Another set of mice (n=3/group) were euthanized 5 days after MCAO and ischemic brain tissue from the ischemic border (IBZ) was extracted for Western blot assay.

**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. The total number of vWF-positive vessels per square millimeter area is presented.

**Histological and Immunohistochemical Assessment**

The brains were fixed by transcardial perfusion with saline, sectioned, tissue processed, and lesion volume calculated as previously described.

For immunostaining, a standard paraffin block was obtained from the center of the lesion (bregma −1 mm to +1 mm). A series of 6-µm thick sections was cut from the block. Every tenth coronal section for a total 5 sections was used for immunohistochemical staining. Antibody against BrdU (1:100; Boehringer Mannheim, Indianapolis, Ind), von Willebrand Factor (vWF, an EC marker, 1:400; Dako, Carpinteria, Calif), and occludin (mouse monoclonal IgG antibody, 1:200 dilution; Zymed) were used. Control experiments consisted of staining brain coronal tissue sections as outlined previously, but nonimmune serum was substituted for the primary antibody. The immunostaining analysis was performed by an investigator blinded to the experimental groups.

**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.

**Foot-Fault Functional Test**

The foot-fault test was performed before MCAO and at 1, 7, and 14 days after MCAO by an investigator who was blinded to the experimental groups. The percentage of foot-faults of the left paw to total steps was determined.

**Histological and Immunohistochemical Assessment**

The brains were fixed by transcardial perfusion with saline, sectioned, tissue processed, and lesion volume calculated as previously described.

**Angiogenesis Measurement**

**Brain EC Proliferation**

Five slides from BrdU-immunostained coronal sections were digitized using a 20× objective (Olympus BX40) through the MCID computer imaging analysis system (Imaging Research, St Catharines, Canada). The number of BrdU-immunoreactive ECs within a total of 10 enlarged and thin-walled vessels located in the IBZ area were counted. Data are presented as the percentage of the number of BrdU-immunoreactive cells within 10 vessels/total EC number.

**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. Intravenous injection of reconstituted HDL (rHDL) significantly augments blood flow recovery and increases capillary density in the ischemic leg. Patients with stroke exposed to power-frequency electromagnetic fields, which increase HDL-C, show a statistically significant better prognosis compared with the control group. 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.
eNOS. 4 HDL-C promotes eNOS activity by maintaining the lipid environment in caveolae where eNOS is colocalized with partner signaling molecules. 5 Enhanced p-eNOS induces a broad range of effects, including the promotion of angiogenesis and mural cell recruitment to immature angiogenic sprouts. 6 EC-derived nitric oxide induces mural cell recruitment as well as subsequent morphogenesis and stabilization of angiogenic vessels. 7 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. 8

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.

Acknowledgments
We thank Qinge Lu for technical assistance.

Sources of Funding
This work was supported by National Institute on Aging RO1 AG031811, National Institute of Neurological Diseases and Stroke RO1 NS047682 and PO1 NS23393, and American Heart Association grant 0750048Z.

Disclosures
None.

References


eNOS Mediates TO90317 Treatment-Induced Angiogenesis and Functional Outcome After Stroke in Mice
Jieli Chen, Xu Cui, Alex Zacharek, Cynthia Roberts and Michael Chopp

Stroke. 2009;40:2532-2538; originally published online May 14, 2009;
doi: 10.1161/STROKEAHA.108.545095

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/40/7/2532

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/