The Neurorestorative Benefit of GW3965 Treatment of Stroke in Mice

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Background and Purpose—GW3965, a synthetic liver X receptor agonist, elevates high-density lipoprotein cholesterol and has antiatherosclerosis and anti-inflammation properties. We tested the hypothesis that GW3965 treatment of stroke increases vascular remodeling, promotes synaptic protein expression and axonal growth in the ischemic brain, and improves functional outcome in mice.

Methods—Mice were subjected to transient middle cerebral artery occlusion and treated without or with different doses of GW3965 (5, 10, or 20 mg/kg) starting 24 hours after middle cerebral artery occlusion daily for 14 days. Neurological functional tests, blood high-density lipoprotein cholesterol measurement, and immunostaining were performed. Mouse brain endothelial cells, primary cultured artery explants, and primary cortical neurons cultures were also used in vitro.

Results—GW3965 treatment of stroke significantly increased blood high-density lipoprotein cholesterol level, synaptic protein expression, axonal density, angiogenesis and arteriogenesis, and Angiopoietin1, Tie2, and occludin expression in the ischemic brain and improved functional outcome compared with middle cerebral artery occlusion control animals (n=10; P<0.05). In vitro, GW3965 and high-density lipoprotein cholesterol also significantly increased capillary-like tube formation and artery explant cell migration as well as neurite outgrowth. Inhibition of Angiopoietin1 attenuated GW3965-induced tube-formation, artery cell migration, and neurite outgrowth (n=6 per group; P<0.05).

Conclusions—These data indicate, for the first time, that GW3965 promotes synaptic protein expression and axonal growth and increases vascular remodeling, which may contribute to improvement of functional outcome after stroke. Increasing Angiopoietin1/Tie2 signaling activity may play an important role in GW3965-induced brain plasticity and neurological recovery from stroke. (Stroke. 2013;44:153-161.)

Key Words: Angiopoietin-1 ■ axonal transport ■ HDL cholesterol ■ liver X receptor ■ stroke

Stroke is a major cause of cerebral white matter and vascular damage, which induces long-term disability as a result of the limited axonal regeneration (axon-regrowth or sprouting) and vascular remodeling (neovascularization and vascular stabilization) in the inhibitory environment of the adult mammalian central nervous system. Successful axonal outgrowth in the adult is central to the process of nerve regeneration and brain repair. Vascular remodeling plays an important role in neurological functional recovery after stroke. Thus, there is a compelling need to develop pharmacological therapeutic approaches specifically designed to reset vascularization and to promote brain plasticity (neurorestoration) to improve neurological deficits after stroke beyond the hyper-acute phase of ischemia.

Liver X receptor (LXR) activates reverse cholesterol transport and raises high-density lipoprotein cholesterol (HDL-C). Increasing HDL-C improves functional outcome after stroke. Treatment of stroke in rats with Niacin, the most effective medication in current clinical use for increasing HDL-C, significantly increases blood HDL-C and improves functional outcome.

Treatment of stroke in mice with T0901317, an agonist of LXRα, increases serum HDL-C as well as improves functional outcome. However, high doses of Niacin produce adverse side effects of skin flushing, stomach upset, and liver damage, and T0901317 concurrently increases total blood cholesterol and triglycerides and may induce severe liver damage. In contrast, GW3965, a synthetic LXRβ selective agonist, raises HDL-C but without inducing hepatic steatosis and hypertriglyceridemia in rodents. Treatment stroke with GW3965 from early-onset (10 minutes to 2 hours) induces neuroprotection by antineuroinflammation and stabilizes the blood–brain barrier (BBB) integrity in the ischemic brain. However, many neuroprotective treatments have failed in clinical trials because stroke patients are very rarely treated within minutes of stroke onset. In this study, we test the effect of GW3965, as a subacute treatment (24 hours after stroke), on HDL-C and functional outcome and the mechanisms underlying the restorative response of brain to this drug on axonal outgrowth and vascular remodeling in a preclinical stroke model in mice.
**Materials and Methods**

All experiments were conducted in accordance with the standards and procedures of the American Council on Animal Care and Institutional Animal Care and Use Committee of Henry Ford Health System.

**Animal Model and Experimental Group**

Adult male C57BL/6J mice aged 2 to 3 months (Charles River) were subjected to 2.5 hours of right middle cerebral artery occlusion (MCAo) by a filament method. Mice were gavaged starting 24 hours after MCAo with the following: (1) saline for vehicle control; (2) different doses of GW3965 (Sigma, 5, 10, or 20 mg/kg) daily for 14 days. All mice received bromodeoxyuridine (BrdU, 50 mg/kg, Sigma) intra-peritoneal injections to label proliferating cells starting 24 hours after MCAo and daily for 14 days. The blood level of HDL-C, total cholesterol (T-CH), and triglyceride, lesion volume calculation, immunostaining, Western blot, and real-time PCR (RT-PCR) were performed 14 days after MCAo. An additional 2 mice were euthanized 24 hours after MCAo to harvest artery explants for the cell migration assay.

**Functional Test**

Modified neurological severity score (mNSS) and left foot-fault tests were performed before MCAo and at 1, 7, and 14 days after MCAo, as previously described.6,7

**HDL-C, T-CH, and Triglyceride Measurement**

Blood levels of HDL-C, T-CH, and triglyceride were measured at 14 days after MCAo using CardioChek P•A analyzer and HDL-C, T-CH, and Triglyceride Measurement were performed before MCAo and at 1, 7, and 14 days after MCAo, as previously described.6,7

**Histological and Immunohistochemical Assessment and Lesion Volume Measurement**

The brains were fixed by transcardial perfusion with saline followed by 4% paraformaldehyde before being embedded in paraffin. The cerebral tissues were cut into 7 equally spaced (1 mm) coronal blocks. A series of adjacent 6-µm-thick sections were cut from each block and stained with hematoxylin and eosin (H&E) for the lesion volumes calculation, as previously described.5-7 Every 10th coronal section cut from the center of the lesion (bregma –1 mm to +1 mm) for a total 5 sections was used for immunohistochemical staining. Immunostaining for Synaptophysin (1:1000, Chemicon), Amyloid precursor protein (APP, 1:50, Cell Signaling Technology), Angiopoietin1 (Ang1, 1:2000, Abcam), von Willebrand Factor (vWF, 1:400, Dako), alpha smooth muscle actin (α-SMA, 1:800, Dako), and histochemical-staining for Bielschowsky silver and Luxol Fast Blue, single immunofluorescent-staining for SM131 (1:1000, Covance), Tie2 (1:80, Santa Cruz Biotechnology), and occludin (1:200, Zymed) conjugated with Cy3 (1:200, Jackson Immunoresearch Laboratories), and double immunofluorescent-staining for BrdU (1:100, Boehringer Mannheim) with vWF or α-SMA were used. Control experiments consisted of staining brain coronal tissue sections as outlined above, but nonimmune serum was substituted for the primary antibody.

**Photo Acquisition and Immunostaining Quantitation**

Images were acquired from 5 slides each brain, with each slide containing 8 fields view within the cortex and striatum from the ischemic boundary zone (IBZ, Figure 2A) and analyzed with a Micro Computer Imaging Device (MCID) imaging analysis system (Imaging Research), as previously described.5-7

The following were calculated in the IBZ: (1) the percentage of Synaptophysin- or Ang1-positive area in the cortex; (2) the percentage of APP, Bielschowsky silver-, SM131-, or Luxol Fast Blue-immunoreactive area in the bundles of the striatum; (3) the percentage of Tie2- or occludin-positive area in vessels; (4) the vascular density by the total number of vWF-vessels per mm²; the average vascular perimeter (µm) from a total of 20 enlarged thin walled vessels; (5) the arterial density by the total numbers of αSMA-arteries with regard to small and large vessels (mean diameter ≥10 µm) per mm²; (6) the average arterial diameter from 10 largest arteries; (7) for cell proliferation, the percentage of BrdU-positive endothelial cells (EC) and smooth muscle cells (SMC) in the vessels and arteries.

**Primary Cortical Neuron and Neurite Outgrowth Measurements**

Primary cortical neurons (PCNs) were subjected to 1 hour of oxygen and glucose deprivation followed by 24 hours of reperfusion.6,7 The hypoxic PCNs were then treated with (n=6 wells per group) the following: (1) nontreatment for control; (2) Ang1 100 ng/ml (mouse Ang1 peptide, Millipore); (3) HDL 80 µg/ml (Calbiochem); (4) GW3965 1 µM; (5) GW3965 1 µM + Anti-Ang1 (1 µg/ml, Rabbit anti-Ang1 affinity purified polyclonal antibody, Millipore) for 24 hours. Then, the PCN cultures were performed TUN1-staining (a phenotypic marker of neural cells, 1:1000, Covance) with Cy3 for neurite outgrowth measurement. Photomicrographs at x20 were captured, and neurite length was measured and averaged.

**Mouse Brain EC Culture and Capillary-Like Tube Formation Assay**

Mouse brain ECs (MBECs; 2×10⁴ cells, ATCC, CRL-2299) were incubated in DMEM medium and were randomly divided into (n=6 wells per group) the following: (1) Nontreatment for control; (2) Ang1 100 ng/ml; (3) HDL 80 µg/ml; (4) GW3965 1 µM; (5) GW3965 1 µM + Anti-Ang1 1 µg/ml treatment for 5 hours. Capillary-like tube formation was quantitated.6

**Primary Artery Explant Culture and Artery Cell Migration Measurement**

The ipsilateral common carotid arteries were surgically removed from mice 24 hours after MCAo. The common carotid arteries were cut into 1 mm² and randomly divided into 5 groups as follows: (1) Nontreatment for control; (2) Ang1 100 ng/ml; (3) HDL 80 µg/ml; (4) GW3965 1 µM; (5) GW3965 1 µM + Anti-Ang1 1 µg/ml. The artery explants were placed in the center of Matrigel and the arterial cultures were allowed to grow for 5 days before being photographed, and the 10 longest distances of neurite outgrowth were measured under a microscope at x4 magnification and averaged (n=6 wells per group).

**RT-PCR**

The ipsilateral brain tissue and MBECs were harvested, total RNA was isolated, and quantitative PCR was performed.6,7 The following primers for RT-PCR were designed using Primer Express software (ABI). GAPDH: Fwd, AGA ACA TCA TCC CTG CAT CC; Rev: CAC ATT GGG GGT AGG AAC AC. Ang1: Fwd, TAT TTT GTG ATT CTG GTG ATT; Rev: GTT TCG CTT TAT TTT GTG AATG. Tie2: Fwd, CGG CCA GGT ACA TAG GAG GAA; Rev, TCA CAT CTC CGA ACA ATC AGC.

**Western Blot**

Equal amounts of cell lysate were subjected to Western blot.6,7 The following primary antibodies were used: anti-Ang1 (1:2000, Abcam), anti-Synaptophysin (1:1000, Chemicon), anti-β-actin (1:2000; Santa Cruz).

**Statistical Analysis**

Independent 2-sample t test was used to assess the lesion volume, immunostaining, Western blot, and RT-PCR measurement. Pearson partial correlations after bivariate correlation were used to analyze the correlation of the blood HDL level with the neurofunctional functional outcome. One-way ANOVA and Tukey test after post hoc test were performed for functional outcome, HDL-C, T-CH, and triglyceride.
neurite outgrowth, tube-formation, and artery explant cell migration analysis. All data are presented as mean±SE. All measurements and functional evaluations were performed in a blinded manner.

Results
GW3965 Treatment of Stroke Increases Blood HDL-C Level and Improves Neurological Outcome
No significant benefit was detected in the 5 mg/kg GW3965 treatment group compared with the MCAo control group. However, 10 mg/kg and 20 mg/kg of GW3965 treatment significantly improved mNSS and left foot-fault 14 days after MCAo. Moreover, 10 mg/kg of GW3965 treatment significantly decreased mNSS score 7 days after MCAo compared with MCAo-control or 5 mg/kg of GW3965 treatment group (Figure 1A, P<0.05, n=10/group). Therefore, in this study, we selected 10 mg/kg as the optimal treatment dose for lesion volume measurement, immunostaining, Western blot, and RT-PCR assay.

Figure 1B show that 10 mg/kg and 20 mg/kg GW3965-treatment of stroke significantly increased blood HDL-C level (average increased 18.9 mg/dl) but did not significantly increase triglyceride level. However, we found that 10 mg/kg and 20 mg/kg GW3965 significantly increased T-CH level

![Figure 1](image_url)

**Figure 1.** GW3965 treatment increases HDL-C levels and improves functional outcome in mice 14 days after MCAo. **A**, mNSS and left foot-fault test. **B**, HDL-C, triglyceride, and T-CH in blood. **C**, Correlation analysis between HDL-C and mNSS or foot-fault. n=10 per group. HDL-C indicates high-density lipoprotein cholesterol; MCAo, middle cerebral artery occlusion; mNSS, modified neurological severity score; and T-CH, total cholesterol.
(average 127.1±5.2) compared with MCAo-control animals (107.8±3.6; \( P=0.028 \)). To investigate the cause of GW3965 treatment–induced increase in T-CH, we subtracted HDL-C from T-CH and found that there is no significant difference in T-CH level after subtraction of HDL-C (after subtraction of HDL-C level, MCAo-control: 54.8±2.84; GW3965-treatment: 58.0±5.31; \( P=0.69 \)). Therefore, the data indicate that the increased T-CH is attributed to the increase of HDL-C. Correlation analysis (Figure 1C) showed that the level of blood HDL is significantly negatively correlated with mNSS score (\( r=-0.899; \ P<0.01 \)) and the percentage of left foot-fault (\( r=-0.764; \ P<0.05 \)). These data suggest that GW3965 treatment of stroke increases HDL-C and thereby improves functional outcome.

**Lesion Volume**

No significant differences of lesion volumes in 10 mg/kg GW3965-treatment (16.11%±1.22%) were detected compared with MCAo-control (17.96%±1.86%; \( P=0.419, \ n=10 \) per group).

**GW3965 Treatment of Stroke Decreases Axon Damage and Increases Synaptic Protein Expression and Axon Density**

Figure 2B–2D shows that GW3965 treatment significantly increased Synaptophysin (a marker for presynaptic plasticity and synaptogenesis) positive area in the IBZ, the density of Bielschowsky silver (a marker for axons), SM131 (a marker of nondamaged phosphorylated neurofilament), and LBF (a myelin marker) but decreased APP (a marker of axonal damage) positive area in the striatal bundles compared with MCAo-control (\( n=10; \ P<0.05 \)). Western blot assay also showed GW3965 treatment significantly increased the protein level of Synaptophysin in the IBZ (\( n=4; \ P<0.05 \)). These data suggest that GW3965 treatment decreases axon damage, increases axon, neurofilament, and myelin densities in the striatal bundles, and promotes synaptic protein expression in the ischemic brain after stroke.

**GW3965 Treatment Increases Angiogenesis, Arteriogenesis, and Vascular Stabilization in the Ischemic Brain**

Figure 3 shows that compared with MCAo-control, GW3965 treatment significantly increased the following: (1) the vascular density and perimeter of vWF-vessels; (2) the arterial density and diameter of \( \alpha \)SMA-arteries; (3) the percentage of BrdU-ECs in vessels and BrdU-SMCs in arteries; and (4) the expression of occludin (a tight junction protein of critical component of BBB) in the IBZ (\( n=10; \ P<0.05 \)). These data indicate that GW3965 treatment increases neovascularization (angiogenesis and arteriogenesis) and vascular stabilization in the ischemic brain.

**Figure 2.** GW3965 treatment increases Synaptophysin expression and axonal and myelin growth and decreases axon damage in the IBZ 14 days after MCAo. **A**, Schematic map showing the IBZ and quantified regions. **B**, Synaptophysin-immunostaining, Western blot, and quantitative data. **C**, Bielschowsky silver and SM131 immunostaining and quantitative data. **D**, LFB and APP immunostaining and quantitative data. Scare bar, 100 μm. \( n=10 \) per group in immunostaining, \( n=4 \) per group in Western blot. APP indicates Amyloid precursor protein; IBZ, ischemic boundary zone; LFB, Luxol Fast Blue; and MCAo, middle cerebral artery occlusion.
GW3965 Treatment Increases Ang1/Tie2 Expression in the Ischemic Brain

GW3965 treatment significantly increased Ang1 and Tie2 expression measured by immunostaining in the IBZ compared with MCAo control (Figure 4A and 4B; n=10; P<0.05). In addition, GW3965 treatment significantly increased Ang1 protein expression analyzed by Western blot and Ang1/Tie2 mRNA level measured by RT-PCR in the IBZ (Figure 4C and 4D; n=4; P<0.05).

GW3965 Increases Neurite Outgrowth, Capillary-Like Tube Formation, and Artery Explant Cell Migration In Vitro

Figure 5 shows that compared with nontreatment control, Ang1 and HDL and GW3965 treatment significantly increased the following: (1) the neurite outgrowth in the hypoxic PCNs; (2) the capillary-like tube formation in the cultured MBECs; and (3) the artery explant cell migration in the primary cultured arteries. However, anti-Ang1 significantly
attenuated GW3965-induced neurite outgrowth, capillary tube formation, and artery explant cell migration (n=6; P<0.05). Consistent with the in vivo data, HDL and GW3965 significantly increased Ang1 mRNA expression, and GW3965 significantly increased Tie2 mRNA expression in the cultured MBECs (n=6; P<0.05).

Discussion

HDL-C is related to stroke recovery. Low level of HDL-C predicts high mortality and rapidly progressive stroke;4,16 Higher levels of HDL-C are associated with better cognitive recovery after stroke.2 LXR s belong to the nuclear receptor superfamily that can regulate important lipid metabolic pathways.10 GW3965 increased expression of the reverse cholesterol transporter ABCA1 and increased the plasma concentrations of HDL-C.9,10 In this study, we found that GW3965 treatment significantly increases blood HDL-C level and improves functional outcome after stroke, and the increased HDL-C is significantly correlated with functional outcome. Therefore, increasing HDL-C by GW3965 treatment may contribute to functional outcome.

Stroke-induced white matter injury may explain the failure of neuroprotective drugs in clinical trials for stroke because these drugs were rarely characterized for their ability to protect white matter.17 Cellular cholesterol modulates axon and dendrite outgrowth and neuronal polarization under culture
conditions.\textsuperscript{18,19} LXR\textsubscript{R}s are essential for maintenance of motor neurons in the spinal cord and dopaminergic neurons in the substantia nigra.\textsuperscript{20} LXR\textsubscript{B} regulates the formation of superficial cortical layers and migration of later-born neurons.\textsuperscript{21} LXR\textsubscript{B} knockout mice exhibit excessive lipid deposits, proliferation of astrocytes, loss of neurons and their dendrites, and disorganized myelin sheaths.\textsuperscript{22} LXR\textsubscript{B} activators induce neuronal differentiation in rat pheochromocytoma cells and stimulate neurite outgrowth.\textsuperscript{23} We found that GW3965 treatment of stroke significantly decreased APP expression in the ischemic brain. APP is a transmembrane glycoprotein that is widely expressed in mammalian tissues and is transported through axons. Axonal damage evokes a disturbance of fast axonal transport, can occur in the early stage of white matter lesions, and cannot transport APP.\textsuperscript{24} Therefore, the decrease of APP expression by GW3965 treatment of stroke reflects the decreased axonal damage in the ischemic brain. Axonal plasticity parallels functional recovery after cortical injury, including stroke. Our previous studies have shown that T0901317 and Niacin significantly increase Synaptophysin expression and improve functional outcome after stroke both in mice and rats.\textsuperscript{5,15} Here, we demonstrate for the first time that GW3965 treatment starting at 24 hours after MCAo significantly increases Synaptophysin expression and axon, myelin, and neurofilament density in the ischemic brain. In addition, GW3965 increases neurite outgrowth in the PCNs. GW3965-induced axonal plasticity may contribute to functional improvement after stroke. A caveat of this study is that we only performed morphological indices of synaptic protein (Synaptophysin) and axonal (Bielschowsky Silver) structural changes. Electrophysiological measurements of axonal and synaptic plasticity warrant further investigation.

Recovery of neurological function after stroke is mediated by many coupled events, including neurogenesis, synaptogenesis, and vascular remodeling.\textsuperscript{1,5,6,14,15} The BBB contributes to the maintenance of brain cholesterol metabolism and protects this uniquely balanced system from exchange with plasma lipoprotein cholesterol.\textsuperscript{25} GW3965 treatment increases occludin expression in vessels in the ischemic brain, which is consistent with previous findings that GW3965 maintains HDL-C homeostasis at the BBB and stabilizes the BBB.\textsuperscript{13} Angiogenesis involves the capillary sprouting, branching, splitting, and differential growth of vessels in the primary plexus to form the mature vascular system. Brain capillary ECs, representing a physiological barrier to the central nervous system, express apolipoprotein A-I, the major HDL-C, and promote cellular cholesterol mobilization.\textsuperscript{25} HDL-C decreases platelet aggregation and inhibits EC apoptosis.\textsuperscript{5} HDL-C also enhances EC migration and angiogenesis.\textsuperscript{26} Intravenous injection of reconstituted HDL stimulates differentiation of endothelial progenitor cells and enhances ischemia-induced angiogenesis.\textsuperscript{27} Arteriogenesis during neovascularization, supporting cells such as pericytes and SMCs are recruited to the vessels to provide structural support and stability for the vascular walls.\textsuperscript{28} LXR knockout mice exhibit enlarged brain blood vessels with weak staining of \textit{s}SMA and excessive lipid accumulation around the abnormal vessels, which lose
their contractile ability and are susceptible to rupture.22 In this study, GW3965 treatment of stroke induces angiogenesis and arteriogenesis identified by increasing EC/SMC proliferation and vascular density/perimeter/diameter in vessels in the ischemic brain. GW3965 also increases MBEC capillary-like tube formation and artery cell migration in vitro. GW3965 treatment–induced angiogenesis/arteriogenesis may contribute to the functional outcome after stroke.

Ang1, an angiopoietic factor, and its receptor Tie2 play an important role in neovascularization. Ang1 also promotes synaptic plasticity and axon remodeling.29 Ang1 stimulates neuronal differentiation and supports neurite outgrowth and synaptogenesis in neuronal progenitor cells, sensory neurons, and PC12 cells.30,31 Niacin increases Ang1 gene and protein expression after stroke.16 Here, GW3965 treatment increases Ang1/Tie2 protein expression in the ischemic brain and Ang1/ Tie2 mRNA expression in cultured MBECs. Ang1 also promotes GW3965-induced capillary-like tube formation, artery explant cell migration, and neurite outgrowth in vitro, which in concert indicate that the Ang1/Tie2 pathway mediates GW3965-induced brain plasticity after stroke.

Conclusions

We demonstrate the neurorestorative benefits of GW3965 in stroke treatment. GW3965 treatment starting 1 day after stroke did not decrease lesion volume but did increase synaptic protein expression, axonal growth, and vascular remodeling in the ischemic brain as well as improves functional outcome. Increasing HDL and upregulation of Ang1/Tie2 activity appears to contribute to the GW3965-induced brain plasticity after stroke.

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Disclosures

None.

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