Niacin Treatment of Stroke Increases Synaptic Plasticity and Axon Growth in Rats

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Background and Purpose—Niacin is the most effective medication in current clinical use for increasing high-density lipoprotein cholesterol. We tested the hypothesis that niacin treatment of stroke promotes synaptic plasticity and axon growth in the ischemic brain.

Methods—Male Wistar rats were subjected to 2 hours of middle cerebral artery occlusion and treated with or without Niaspan (a prolonged-release formulation of niacin, 40 mg/kg) daily for 14 days starting 24 hours after middle cerebral artery occlusion. The expression of synaptophysin, Nogo receptor, Bielschowsky silver, brain-derived neurotrophic factor, and its receptor tropomyosin-related kinase B were measured by immunohistostaining and Western blot, respectively, in the ischemic brain. Complementing in vivo studies, primary cultured neurons were used to test the effect of niacin and high-density lipoprotein on neurite outgrowth and brain-derived neurotrophic factor/tropomyosin-related kinase B expression.

Results—Niaspan treatment of stroke significantly increased synaptophysin, Bielschowsky silver, brain-derived neurotrophic factor/tropomyosin-related kinase B expression, and decreased Nogo receptor expression in the ischemic brain compared with middle cerebral artery occlusion control animals (P<0.05, n=8/group). Niacin and high-density lipoprotein treatment significantly increased neurite outgrowth and brain-derived neurotrophic factor/tropomyosin-related kinase B expression in primary cultured neurons. Tropomyosin-related kinase B inhibitor attenuated niacin-induced neurite outgrowth (P<0.05, n=6/group).

Conclusions—Niacin treatment of stroke promotes synaptic plasticity and axon growth, which is mediated, at least partially, by the brain-derived neurotrophic factor/tropomyosin-related kinase B pathways. (Stroke. 2010;41:00-00.)

Key Words: axon growth | HDL-cholesterol | niacin | plasticity | stroke | synaptic

Synaptic plasticity and axon growth are related to behavioral change and functional recovery after brain and spinal cord injury.1 Functional alterations in motor cortex organization are accompanied by changes in dendritic and synaptic structure.2 Cortical stimulation-induced functional improvements after stroke are mediated by synaptic structural plasticity.3 Increases in dendritic arborization and spine density are potential morphological strategies that enable the brain to reorganize its neuronal circuits.4

High-density lipoprotein cholesterol (HDL-C) is critical in maintaining the homeostasis of cell membrane cholesterol and thus plays an essential role in the regulation of synaptic function and cell plasticity.5 The expression of brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) supports neuron survival and axon growth after neuronal injury.6-7 Voluntary exercise leads to an endogenous upregulation of BDNF and associated proteins involved in synaptic function and enhances functional recovery after traumatic brain injury.8 Synaptic plasticity is also influenced by mutations in BDNF.9

Niacin (nicotinic acid) is the most effective medication in current clinical use for increasing HDL-C.10 Our previous study showed that Niaspan (a prolonged-release formulation of niacin) treatment of stroke significantly increases serum HDL-C level and improves functional outcome in rats.11 However, whether niacin increases synaptic plasticity and axon growth and whether BDNF/TrkB play a role in niacin-induced synaptic plasticity and axon growth has not been investigated. In this study, we tested a novel hypothesis, that niacin treatment of stroke promotes synaptic plasticity and axon growth in the ischemic brain in rats. In addition, the contributions of BDNF/TrkB to niacin-induced synaptic plasticity and axon growth were investigated.

Materials and Methods

Animal Middle Cerebral Artery Occlusion and Experimental Groups

Adult male Wistar rats weighing 270 to 300 g (Jackson Laboratory, Bar Harbor, Maine) were subjected to 2 hours of right middle cerebral artery occlusion by a microsurgical technique and then treated with or without Niaspan (40 mg/kg) daily for 14 days starting 24 hours after occlusion.
cerebral arterial occlusion (MCAO). Rats were gavaged starting 24 hours after surgery with: (1) saline for vehicle control; or (2) 40 mg/kg Niaspan (KOS Pharmaceuticals) daily for 14 days. Sham-operated rats underwent the same surgical procedure without suture insertion. These rats were euthanized 14 days after MCAO for immunostaining (n=8/group). Additional sets of rats (n=4/group) were euthanized 5 days after MCAO, and the brain tissues were prepared for Western blot assay.

Histological and Immunohistochemical Assessment
Rats were euthanized 14 days after stroke. The brains were fixed by transcardial perfusion with saline followed by perfusion and immersion in 4% paraformaldehyde before being embedded in paraffin. 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 were cut from the block. Every 10th coronal section for a total 5 sections was used for immunohistochemical staining. Antibody against Synaptophysin (1:1000; Chemicon), Nogo receptor (NgR; 1:50; Santa Cruz), BDNF (1:300; Santa Cruz), and TrkB (1:500; Santa Cruz) immunostaining was performed. Bielschowsky silver staining was also used as previously described. 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.

Immunostaining Quantification
Like our previous description, synaptophysin, NgR, BDNF, TrkB, and Bielschowsky silver immunohistostained sections were digitized using a 40× objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with the Micro Computer Imaging Device computer imaging analysis system (Imaging Research). Synaptophysin-, BDNF-, and TrkB-positive area was counted in the ischemic boundary zone (IBZ, which adjacent to the ischemic core) in each section. Five sections and 8 views in each section were counted per rat. For semiquantitative measurements of Bielschowsky silver and NgR, the positive stained areas in the bundle of striatum in the IBZ were measured. Data were analyzed in a blinded manner and presented as percentage of positive area for synaptophysin, Bielschowsky silver, NgR, BDNF, and TrkB immunoreactivity, respectively.

Primary Cultured Neuron and Treatments
To test whether niacin regulates dendrite outgrowth, primary cultured neuron (PCN) culture was used. Embryonic Day 17 cortical cells were isolated under the 10× microscope from embryonic brain of Wistar rats and cultured in 4-chamber slides with Neurobasal-A medium (GIBCO) containing 2% B27 medium supplement (GIBCO) and antibiotics for 7 days (6 chambers/group) in vitro. To mimic the ischemic condition in vivo, oxygen-glucose deprivation (OGD) was induced in vitro as previously described. OGD was induced within an anaerobic chamber. Briefly, the PCN cultures were transferred to the anaerobic chamber (Model 1025; Forma Scientific) and were incubated in 85% N2, 10% H2, and 5% CO2 at 37°C for 1 hour. The PCN cultures were removed from the anaerobic chamber, rinsed with phosphate-buffered saline, and fed with the primary media and the groups were divided into: (1) control; (2) high-density lipoprotein (HDL; 80 μg/mL; Calbiochem, Cat. #437641); (3) 1 mmol/L niacin; (4) 5 mmol/L niacin; (5) TrkB inhibitor (K252a, 200 nmol/L; Calbiochem, Cat. #480354); (6) TrkB inhibitor+HDL; (7) TrkB inhibitor+1 mmol/L niacin; or (8) TrkB inhibitor+5 mmol/L niacin for 24 hours. Western blot assay and real time-polymerase chain reaction (RT-PCR) were performed, respectively. The PCN cultures were also performed for neuron-specific Class III β-tubulin (TUJ1, a phenotypic marker of neural cells) immunofluorescent staining using a monoclonal anti-TUJ1 antibody (Covance; 1:1000) with Cy3 for PCN number counting and neurite outgrowth measurement.

Quantification of PCN Numbers and Measurement of PCN Neurite Outgrowth
To count PCN numbers and trace the axonal arbor of fluorescently labeled neurons, the fluorescent photomicrographs were captured at 40× magnification with a digital camera; the TUJ1-positive PCN numbers and the length of TUJ1-positive dendrites were measured using Micro Computer Imaging Device analysis system. The average number of TUJ1-positive PCNs per 40× field and the average length of total 20 neuronal dendrite outgrowth were presented.

Western Blot
Rats were euthanized 5 days after MCAO and brain tissues were extracted from the IBZ tissue. Equal amounts of cell lysate were subjected to Western blot analysis, as previously described. PCN cells were harvested after 24 hours of treatment for Western blot. Total protein was isolated from treated cells with TRIzol (Invitrogen) following a standard protocol. Heat the protein samples in 1% sodium dodecyl sulfate for approximately 20 minutes at 60°C to recover the protein activity. Specific proteins were visualized using a SuperSignal West Pico chemiluminescence kit (Pierce). The following primary antibodies were used: anti-BDNF (1:1000; Santa Cruz), anti-TrkB (1:1000; Santa Cruz), and anti-β-actin (1:2000; Sigma).

Real-Time Polymerase Chain Reaction
PCN cultures were harvested after 24 hours of treatment and total RNA was isolated with TRIzol (Invitrogen). Quantitative PCR was performed using the SYBR Green RT-PCR method on an ABI 7000 PCR instrument (Applied Biosystems), as previously described. The following primers for RT-PCR were designed using Primer Express software (ABI). BDNF forward: TAC TTC GGT ATG ATG ACG GCC; reverse: GTC AGA CTC GAA GCT GCC. TrkB forward: TCA TCA AGT CAG AGG TGA CAG G; reverse: ACT GGG TAC ACT CCT TCT CTC G; glyceraldehyde-3-phosphate dehydrogenase forward: AGA ACA TCA TCC CTG CAT CC; reverse: CAC ATT GGG GGT AGG AAC AC. Each sample was tested in triplicate, and samples were obtained from 6 independent experiments that were used for analysis of relative gene expression data using the 2^-ΔΔCT method.

Statistical Analysis
Two-way analysis of variance was performed on data of the percentage of positive area for synaptophysin, NgR, Bielschowsky silver, and NgR immunostaining were performed. Synaptophysin was used for immunohistochemical staining. Antibody against Synaptophysin (1:1000; Chemicon), Nogo receptor (NgR; 1:50; Santa Cruz), BDNF (1:300; Santa Cruz), and TrkB (1:500; Santa Cruz) immunostaining analysis was performed by an investigator blinded to the experimental groups.

Results
Niaspan Treatment of Stroke Increases Synaptic Plasticity and Axon Growth in the Ischemic Brain
To test whether Niaspan treatment of stroke induces synaptic plasticity and axon growth, synaptophysin, Bielschowsky silver, and NgR immunostaining were performed. Synaptophysin is a marker for presynaptic plasticity and synaptogenesis. Bielschowsky silver is a marker for axons. NgR, a neurite outgrowth inhibitor, regulates axonal growth as well as axon regeneration after injury. Figure 1A–L shows that the expression of Bielschowsky silver (Figure 1A–D) and synaptophysin (Figure 1I–L) significantly increased in the
IBZ, whereas the expression of NgR (Figure 1E–H) significantly decreased in the Niaspan treatment rats compared with MCAO control rats ($P<0.05$, $n=8$ /group).

**Niaspan Treatment of Stroke Increases BDNF and TrkB Expression in the Ischemic Brain**

To elucidate the mechanism of Niaspan-induced synaptic plasticity and axon growth, BDNF and TrkB expression in the ischemic brain was measured using immunostaining and Western blot assays. Figure 1M–T shows that Niaspan treatment of stroke significantly increased BDNF (Figure 1M–P) and TrkB (Figure 1Q–T) expression in the IBZ compared with MCAO alone control animals ($P<0.05$, $n=8$ /group).

To verify the immunohistostaining data, rat brain tissues were extracted from Niaspan-treatment and MCAO control rats 5 days after MCAO, and Western blot assays were performed. Figure 2A–C shows that BDNF and TrkB expression in the IBZ significantly increased in Niaspan-treated rats compared with MCAO control rats ($P<0.05$, $n=4$ /group).

**Niacin Increases BDNF and TrkB Expression in PCNs**

To further investigate the mechanism underlying the BDNF/TrkB pathway mediates niacin-induced synaptic plasticity and axon growth, and whether the increased BDNF/TrkB expression is mediated by niacin treatment-induced increase in HDL, the expression of BDNF and TrkB were also investigated using an in vitro PCN culture model. PCNs were subjected to OGD and treated with niacin (1 mmol/L or 5 mmol/L) and HDL (80 μg/mL) for 24 hours. Figure 2D–H shows that niacin (1 mmol/L or 5 mmol/L) and HDL (80 μg/mL) for 24 hours.
μg/mL) treatment significantly increased BDNF and TrkB protein and mRNA expression measured by Western blot assay (Figure 2D–F) and RT-PCR (Figure 2G–H) in PCN cultures compared with the nontreatment control (P<0.05, n=6/group).

Niacin Increases Neurite Outgrowth in PCNs

To test whether niacin/HDL affects PCN cell number and increases neurite outgrowth, and whether the BDNF/TrkB pathway plays a role in niacin/HDL-induced neurite outgrowth, PCN cultures were treated with HDL, 1 mmol/L niacin, 5 mmol/L niacin, and a TrkB inhibitor (K252a; 200 nmol/L) for 24 hours after OGD. TUJ1 immunofluorescent staining, TUJ1-positive cell number, and dendrite outgrowth measurements were performed. Figure 3A–I shows that HDL (Figure 3B), 1 mmol/L niacin (Figure 3C), and 5 mmol/L niacin (Figure 3D) significantly increased neurite outgrowth; TrkB inhibitor (Figure 3E), however, significantly decreased neurite outgrowth in PCNs compared with the nontreatment control (Figure 3A). The TrkB inhibitor significantly de-

Figure 2. Niaspan treatment of stroke increases BDNF/TrkB expression in the ischemic brain, and niacin/HDL increases BDNF/TrkB expression in PCN cultures. A–C, BDNF and TrkB Western blot assay (A) and quantitative data (B: BDNF, C: TrkB) in the IBZ (n=4/group); D–F, BDNF and TrkB Western blot assay and quantitative data (E: BDNF, F: TrkB) in PCNs (n=6/group); G–H, BDNF (G) and TrkB (H) mRNA expression measured by RT-PCR in PCNs (n=6/group).

Figure 3. Niacin increases neurite outgrowth in PCNs after OGD. A–H, Neurite outgrowth in the PCNs (A, control; B, 80 μg/mL HDL; C, 1 mM niacin; D, 5 mM niacin; E, 200 nM TrkB; F, TrkB inhibitor+HDL; G, TrKB inhibitor+1 mM niacin; H, TrKB inhibitor+5 mM niacin); (I) quantitative data of PCN neurite outgrowth (n=6/group). J, Quantitative data of PCN numbers (n=6/group). Scale bar in A=40 μm.
creased HDL- (Figure 3F) and niacin- (Figure 3G–H) induced neurite outgrowth in PCNs compared with the niacin-treated alone group \( (P<0.05, n=6/group) \). Niacin and HDL do not affect PCN number; TrkB inhibitor decreases PCN numbers in niacin-treated PCN compared with niacin alone-treated PCN \( (P<0.05, n=6/group) \). These data indicate that niacin treatment significantly increased neurite outgrowth in PCNs, and niacin-induced neurite outgrowth is, at least partially, mediated by HDL-induced upregulation in BDNF/TrkB.

**Discussion**

Functional recovery after acute central nervous system injury in humans such as stroke is exceptionally limited, leaving the affected individual with lifelong neurological deficits. This lack of functional recovery such as motor recovery can, at least in part, be attributed to the restriction of axon growth and synaptic plasticity.\(^{22-23}\)

 Cellular cholesterol modulates axon and dendrite outgrowth and neuronal polarization under culture conditions.\(^{24-25}\) Astrocytes are a major source of HDL-C synthesis in the central nervous system.\(^{26}\) Growing evidence strengthens the link between brain HDL-C metabolism and factors involved in synaptic plasticity. For example, the scavenger receptor, Class B, Type I, binds HDL and mediates the selective transfer of cholesteryl esters and \( \alpha \)-tocopherol from circulating HDL to cells. Aged scavenger receptor, Class B, Type I knockout mice show deficient synaptic plasticity (long-term potentiation) in the CA1 region of the hippocampus. Very aged scavenger receptor, Class B, Type I knockout mice also display selective impairments in recognition memory and spatial memory.\(^{5}\) The cholesterol transporter ATP-binding cassette transporter A1 (ABCA1) also plays a critical role in brain cholesterol metabolism. Mice that specifically lacked ABCA1 in the central nervous system exhibit reduced plasma HDL-C levels and changes in synaptic ultrastructure, including reduced synapse and synaptic vesicle numbers. Disturbances in cholesterol transport in the central nervous system are associated with structural and functional deficits in neurons.\(^{27}\) Thus, agents that increase HDL level may increase synaptic plasticity and axon growth after stroke. Niacin is the most potent HDL-C-increasing drug used in the clinic. Our previous study showed that Niaspan treatment of stroke significantly decreases NgR mRNA is downregulated in the dentate gyrus after delivery of BDNF into the rat hippocampus formation in rats subjected to kainic acid.\(^{32}\) In the present study, niacin treatment of stroke significantly increases BDNF/TrkB expression both in the ischemic brain and in PCN cultures. HDL also significantly increases the expression in BDNF/TrkB and neurite outgrowth in PCN cultures. In addition, a TrkB inhibitor significantly decreases HDL- and niacin-induced neurite outgrowth, which indicates that the BDNF/TrkB axis may mediate, at least in part, niacin/HDL-induced synaptic plasticity and axon growth.

**Summary**

We demonstrated that niacin treatment of stroke promotes synaptic plasticity and axon growth in rats. The BDNF/TrkB pathways appear to contribute to niacin/HDL-induced synaptic plasticity and axon growth after stroke.

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**Disclosures**

None.

**References**


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