D-4F Decreases White Matter Damage After Stroke in Mice

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Background and Purpose—Stroke-induced neuroinflammation and white matter damage are associated with neurological deficits. Whether D-4F, an apolipoprotein A-I mimic peptide, treatment of stroke decreases neuroinflammation and white matter damage and improves functional outcome has not been investigated.

Methods—Adult male C57BL/6 mice were subjected to permanent middle cerebral artery occlusion (MCAo) and were orally administered saline as a vehicle control and different doses of D-4F (2, 4, 8, 16, or 32 mg/kg) starting at 2 h after MCAo and daily until euthanized at 7 days after MCAo. D-4F treatment did not alter the blood levels of high-density lipoprotein, total cholesterol, triglyceride, blood–brain barrier leakage, and infarction volume compared with control group.

Results—D-4F (16 mg/kg) treatment of stroke significantly improved functional outcome, increased the white matter density and the number of oligodendrocyte progenitor cells in the ischemic boundary zone of the ipsilateral striatum, and increased myelin basic protein, insulin-like growth factor-1 (IGF1), but decreased inflammatory factor Toll-like receptor-4 and tumor necrosis factor-α expression in the ischemic brain 7 days after MCAo (P<0.05, n=11/group). The neurtile/axonal outgrowth in primary cultured neurons was significantly increased when treated with D-4F (100 ng/mL) and IGF1 (100 ng/mL) compared with the nontreatment control. Inhibition of IGF1 significantly attenuated D-4F or IGF1 treatment–induced axonal outgrowth. D-4F-treatment did not increase oligodendrocyte–progenitor cell proliferation but decreased oligodendrocyte–progenitor cell death.

Conclusions—D-4F treatment initiated 2 h after MCAo decreases neuroinflammation and white matter damage and improves functional outcome after stroke. D-4F-induced increase in IGF1 may contribute to D-4F–induced neurite/axonal outgrowth after stroke. (Stroke. 2016;47:214-220. DOI: 10.1161/STROKEAHA.115.011046.)

Key Words: axon • insulin-like growth factor-1 • neuroinflammation • stroke • white matter

Increasing high-density lipoprotein (HDL) functionality may have important implications for treatment and prevention of cerebral inflammation and white matter (WM) damage after stroke. D-4F is an 18-amino acid peptide that mimics the tertiary structure of apolipoprotein A-I and is easily absorbed without liver toxicity. Previous studies show that orally administered D-4F does not increase serum levels of HDL, but does enhance HDL function, such as increased pre-beta HDL formation, increased cholesterol efflux, conversion of proinflammatory and dysfunctional HDL to anti-inflammatory and functional HDL, and alteration of HDL particle size distribution and metabolism. However, the effect of D-4F treatment of stroke on reducing WM damage and improving neurological functional recovery after stroke has not been investigated.

Macrophages/microglia were activated after stroke and produced proinflammatory cytokines, proteolytic enzymes, and growth factors that alter survival of neurons and axonal growth. Toll-like receptors (TLRs) and tumor necrosis factor-alpha (TNFα) are implicated in the stroke-induced inflammatory process. The expression of TLR4 is upregulated in ischemic stroke, at least during the subacute stage, which increases neuroinflammation and exacerbates stroke injury. Insulin-like growth factor-1 (IGF1) expression is regulated by TNFα and has anti-inflammatory properties and an opposite effect to TNFα. Increasing IGF1 promotes neuroprotection and decreases WM damage after stroke. In this study, we investigate the hypothesis that D-4F treatment reduces inflammation, improves WM damage and functional recovery after stroke in mice, at least partially via the IGF1 signaling pathway.

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 Groups

Adult male C57BL/6 mice (3–4 months old; Jackson Laboratory, Bar Harbor, ME) were subjected to permanent right middle cerebral

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artery occlusion (MCAo) using a 6-0 nylon filament method, as previously described. The animals were randomly divided into 3 sets: (1) mice (n=11/group) were randomly orally administered different doses of D-4F (BioMatik, Cambridge, ON, Canada) 2, 4, 8, 16, or 32 mg/kg and saline as vehicle control starting at 2 h after MCAo and daily for a total of 7 days. These mice were used for functional test, blood biochemical and lesional volume measurement, and histochemical and immunohistochemical staining: (2) mice (n=12/group) were orally administered saline or D-4F 16 mg/kg for 7 days and used for blood–brain barrier (BBB) leakage measurement (n=6/group) and Western blot (WB) and real time polymerase chain reaction (RT-PCR) assays (n=6/group). All survival animals were euthanized 7 days after MCAo. Total 120 mice were used and 18 mice died; the mortality rate is 15% in this study.

Functional Test
To evaluate neurological functional deficits and recovery after stroke, modified neurological severity score and left foot-fault test were performed before MCAo and at 1, 3, and 7 days after MCAo. 

Blood Biochemical Measurement
Blood was collected from the tail vein 7 days after MCAo, and the level of HDL, total cholesterol, and triglyceride were measured using CardioChek P•A analyzer (Polymer Technology System Inc. Indianapolis, IN) following the manufacturer’s instruction.

BBB Leakage Measurement
Evans blue dye extravasation was used for quantification of BBB leakage 7 days after MCAo.

Histochemical/Histoimmunostaining and Infarct Volume Measurement
Mice brains were fixed embedded in paraffin and were cut into 7 equally spaced (1 mm) coronal blocks. For calculation of lesion volume, a series of adjacent 6-μm-thick sections were cut from each block and stained with hematoxylin and eosin. Every 10th coronal section was cut from the center of the lesion (bregma −1 mm to +1 mm), and a total of 5 sections were used for staining of Bielschowsky silver (BS, an axon marker), Luxol Fast Blue (a myelin marker), and platelet-derived growth factor receptor alpha+ cells in the IBZ of the ischemic lesion. The IBZ brain tissue and the equal volumes of homologous contralateral tissue were extracted for WB assay. Total protein was isolated with TRIzol (Invitrogen), and the protein samples were heated in 1% SDS for 20 min at 60°C to recover the protein activity. The samples were normalized for total protein content in WB assay. Specific proteins were visualized using a SuperSignal West Pico chemiluminescence kit (Pierce). The following primary antibodies against myelin basic protein (a myelin marker, 1:200; Dako, Carpinteria, CA), IGF1 (1:1000, Abcam), TLR4 (1:500, Santa Cruz), TNFα (1: 1000, Abcam), and β-actin (1: 2000; Santa Cruz) were used for WB assay.

RT-PCR Analysis
Total RNA was isolated using a standard protocol. Quantitative PCR was performed on an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA) 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. The following primers for RT-PCR were designed using Primer Express software (ABI): IGF1: Fwd: TGGATGCTCTCTGGTCTGG, Rev: TGGTAAGTGAGGAGCTGTGATC; TLR4: Fwd: TAT TTT GTG ATT CTG GTG ATT; Rev: GTT TCG CAT TAT TTT GTG AATG; TNFα: Fwd: TACCTCCAGGTTCCTCTCAAGG, Rev: GAGGTGTACTCTCTCTGGCTA; GAPDH: Fwd: AGA AGA TCC CTG CAT CC, Rev: CAC ATT GGG GTT AGG AAC AC.

Primary Cortical Neuron Culture and Axonal Outgrowth Measurement
To investigate whether D-4F treatment increases neurite outgrowth and to further elucidate whether IGF1 mediates D-4F-induced neurite outgrowth after stroke, a primary cortical neuron (PCN) culture and neurite outgrowth measurements were used. Briefly, PCNs were isolated from E15 C57BL/6 mice and cultured with 8-well slide chambers. On day in vitro 3, to mimic ischemia in vivo, the PCNs were subjected to 3 h of oxygen and glucose deprivation in DMEM with serum- and glucose-free media. The PCNs were then cultured with neurobasal medium plus 2% B-27, 2 mmol/L Glutamax, and 1% antibiotic antimycotic. After 24 h, the PCNs were divided into 5 groups as follows: (1) nontreatment as control; (2) +D-4F (100 ng/mL); (3) +IGF1 (recombinant mouse IGF1 protein, 100 ng/mL); (4) +D-4F+IGF1 inhibitor (AG1024, 10 μM; Calbiochem, Cat No 121767); and (5) +IGF1+IGF1 inhibitor. AG1024 is a cell-permeable, reversible, substrate competitive, and specific inhibitor of IGF-1 and insulin receptor tyrosine kinase activity. The PCNs were allowed to incubate for another 24 h before being immunostained for TUJ1 (a phenotypic marker of neural cells, 1:1000; Covance) with Cy3 and photographed using a 10× objective fluorescent microscope (Zeiss). The average length of the 20 longest neurites in each well (n=6 wells/group) was measured using MCID software.

To further investigate whether D-4F treatment increases axonal outgrowth, and whether IGF1 mediates D-4F treatment–induced axonal outgrowth, a microfluidic axonal growth model (Standard Neuron Device; catalog No SND450, Xona Microfluidics) was also used. Briefly, microchambers were affinity fixed with poly-γ-lysine–coated (Sigma-Aldrich) dishes (35 mm, Corning), and the PCNs were plated at a density of 6×103 cells/chamber in DMEM with 5% FBS. PCNs were changed to neuronal growth media after 24 h incubation. On day in vitro 3, culture medium containing 20 μM 5-fluorodeoxyuridine was used to kill astrocytes. The PCN cultures were then divided into the same 5 groups as above and subjected to the above neurite outgrowth measurements.

Premature Oligodendrocyte Cell Line Culture
To investigate the effect of D-4F on OPC proliferation and survival, an immortalized mouse premature oligodendrocyte cell line (N20.1; generously provided by Dr Anthony Camarghoni, UCLA) culture was used. N20.1 cells were cultured in DMEM/F12 (Invitrogen) medium with 10% FBS and 100 μg/mL G418. Two hours of oxygen and glucose deprivation was induced, and cells were treated with (1) nontreatment for control; and (2) D-4F (100 ng/mL) for 48 h (n=6 wells/group). Lactate dehydrogenase (LDH), as a cell death assay, and MTS (3-[4,5-dimethylthiazol-2-yl]-[5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium], as a cell proliferation assay, were performed.

For the LDH assay, the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega) was used. Data are presented as percentage of LDH level in the media to total LDH both in the media and cells. For MTS assay, CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) was used. Absorbance was recorded at 490 nm.
Statistical Analysis

All measurements were performed by experimenters blinded to each group and condition. Results are expressed as the mean±SE. Significance of difference between animal groups was determined by 1-way analysis of variance followed by Tukey’s post hoc test for blood chemistry, lesion volume, functional evaluation, and neurite/axonal outgrowth; 2-way analysis of variance was used for analysis of WB and RT-PCR data; and Student t test was used for BBB leakage, WM density, and OPC number measurements. Correlation between the BS+ axon density and modified neurological severity score was tested by Pearson’s correlation coefficients.

Results

D-4F Treatment Effect on Blood Lipids and Lipoproteins, Lesion Volume, BBB Leakage, and Functional Outcome 7 Days After Stroke

There were no significant differences in the blood levels of HDL, total cholesterol and triglyceride (Figure 1A), and infarction volume (Figure 1B) among the control or MCAo mice treated with 2, 4, 8, 16, and 32 mg/kg of D-4F daily for 7 days. However, treatment of stroke with 16 mg/kg D-4F significantly improved neurological functional outcome 7 days as tested by modified neurological severity score and at 3 and 7 days as tested by foot fault after stroke (P<0.05), respectively. Therefore, the 16 mg/kg D-4F treatment was selected for further measurement of BBB leakage, WM density, and WB/RT-PCR assay. No significant difference in BBB leakage was observed between D-4F treatment and MCAo alone groups 7 days after stroke (Figure 1C).

D-4F Treatment Decreased WM Damage and Increased OPC Number in the Ischemic Brain After Stroke

To test whether D-4F treatment decreases WM damage after stroke, the WM change and OPC number measurements were performed using histochemical- or histoimmuno-staining. The density of BS+axon/Luxol Fast Blue+myelin/SMI31+phosphorylated neurofilament in the WM bundles in the IBZ of ipsilateral hemispheres and the number of platelet-derived growth factor receptor alpha+OPCs in the IBZ of ipsilateral striatum were significantly increased (Figure 2A–2D, P<0.05, n=11/group) in D-4F treatment mice compared with nontreatment MCAo control mice. Using Pearson’s correlation coefficients analysis, we found that the functional outcome (modified neurological severity score) was significantly and negatively correlated with BS+axon density (Figure 2A; r=−0.835, P<0.05).

To confirm the immunostaining data, myelin basic protein level was measured by WB assay and was confirmed increased by D-4F treatment (Figure 3A; P<0.05, n=6/group). These data indicate that increasing axonal density may contribute to D-4F–induced neurological functional outcome after stroke.

D-4F Treatment of Stroke Decreased Inflammatory Factors (TLR4 and TNFα) but Increased IGF1 Expression in the Ischemic Brain After Stroke

To test the mechanism by which D-4F treatment reduced WM damage after stroke, we measured IGF1/TLR4/TNFα gene and protein expression. Our data show that IGF1 protein/mRNA levels are decreased but TNFα/TLR4 protein/mRNA levels significantly increased in the ipsilateral compared with the contralateral brains in MCAo control mice (Figure 3A and 3B, P<0.05, n=6/group). D-4F treatment significantly increased IGF1, but decreased TNFα/TLR4 protein/mRNA levels in the ischemic brain. D-4F treatment also significantly
increased IGF1 but decreased TNFα/TLR4 mRNA levels in the contralateral brain when compared with MCAo control mice (Figure 3B; \( P<0.05 \), \( n=6/\text{group} \)).

**D-4F Treatment Increased PCN Neurite and Axon Outgrowth and Decreased OPC Death; IGF1 Mediates D-4F Treatment–Induced Neurite/Axon Growth After Stroke**

To confirm the in vivo findings, in vitro neurite/axonal outgrowth and OPC proliferation/survival studies were performed using PCN/axonal or N20.1 cell culture models. The optimal doses of D-4F (100 ng/mL) used for PCN and OPC cultures were selected from our pilot study of D-4F dose effect (5, 50, 100, 250, and 500 ng/mL) on the MTS assay (data not shown).

We found that PCN neurite outgrowth significantly increased after treatment with D-4F or IGF1 compared with the nontreatment PCNs 24 h after oxygen and glucose deprivation (Figure 4A; \( P<0.05 \), \( n=6/\text{group} \)). Inhibition of IGF significantly attenuated D-4F- and IGF-induced neurite outgrowth. Similarly, the axonal outgrowth in axon cultures treated both with D-4F and IGF1 significantly increased compared with the nontreatment axons 48 h after treatment (Figure 4B; \( P<0.05 \), \( n=6/\text{group} \)). Inhibition of IGF significantly attenuated D-4F- and IGF-induced axonal outgrowth.

D-4F treatment did not increase OPC proliferation but significantly decreased OPC death measured by MTS and LDH assays, respectively (Figure 4C), which is consistent with the myelin protective effect observed in vivo.

**Discussion**

In this study, we found that D-4F treatment of stroke initiated 2 h after the ischemic onset did not change blood levels of lipid and lipoprotein, infarct volume, and BBB leakage in the ischemic brain, but significantly decreased TLR4 and TNFα gene and protein levels in the ischemic brain and improved neurological functional outcome 7 days after stroke. D-4F treatment patients with coronary heart disease does not change plasma lipid or lipoprotein levels, but improves the HDL.
anti-inflammatory index.\textsuperscript{20} D-4F treatment also had no effect on infarct size in mice with myocardial infarction.\textsuperscript{21} LDL receptor null (LDLR\textsuperscript{−/−}) mice treated with D-4F exhibited no significant differences in plasma lipids and lipoproteins, but had significantly reduced inflammation and cognitive impairment.\textsuperscript{22,23} D-4F inhibits inflammatory properties (TNF\textsubscript{α} and IL-1\textsubscript{β}) in the brain of APPSwe-PS1 Delta E9 mice and improves cognitive function.\textsuperscript{24} These data are consistent with our findings in the present study.

After an ischemic stroke, there is a prolonged inflammatory response and a secondary phase of WM damage that may be more amenable to treatment than the acute injury.\textsuperscript{8,9} WM is composed of bundles of myelinated axons. WM damage defined as axonal degeneration and loss of myelin induces disturbance of nerve impulse transport between neurons and evokes serious neurological functional deficits after stroke.\textsuperscript{25} OPCs are immature forms of oligodendrocytes. OPC may proliferate and differentiate into mature oligodendrocytes and, thereby, help to decrease the burden of axonal injury. Enhanced proliferation, migration, and differentiation of OPCs are seen in the peri-infarct region and in the subventricular zone after stroke.\textsuperscript{26} OPCs are the key source of myelin production and, thus, are essential for repair of damaged WM after stroke and play an important role in promotion of functional recovery after brain injury.\textsuperscript{27} Stroke-induced cerebral inflammatory processes adversely impact WM, including axon and myelin structural integrity, which is associated with long-term neurological functional deficits after stroke.\textsuperscript{28,29} In this study,
we found that the decreased axonal damage was significantly and negatively correlated with the functional outcome. D-4F treatment of stroke increased axonal and myelin integrity as well as increased the numbers of oligodendrocytes/OPCs in the ischemic brain. D-4F also increased PCN neurite/axonal outgrowth and OPC survival in vitro. These data support the hypothesis that by decreasing WM damage, D-4F treatment induced functional outcome after stroke.

IGF1 is an important growth factor that promotes neuronal and oligodendrocytes differentiation, proliferation, myelination, and neurite outgrowth, reduces apoptosis, and sustains cell survival both in the developing brain and throughout life.\textsuperscript{30–35} IGF1 also reduces gray matter and WM damage after stroke and upregulates OPC numbers via suppression of apoptosis.\textsuperscript{33,36,37} Conversely, one might predict that inhibition of IGF1 signaling via blockade of the IGF1 receptor would potentially increase the severity of ischemic brain injury. TNF\textalpha{} markedly decreased IGF1 mRNA and protein, leading to a reduction in bioactive IGF1 in myoblasts, skeletal muscle, and vascular smooth muscle cell.\textsuperscript{38} Activation of TNF\textalpha{} signaling can inhibit IGF1 signaling and thus elevate inflammatory cytokines in skeletal muscle in vivo.\textsuperscript{3,14} In the present study, we found that D-4F significantly decreased TNF\textalpha{} but increased IGF1 level and decreased WM damage in the ischemic brain after stroke, and in vitro study shows that both D-4F and IGF1 treatment significantly increased neurite/axon outgrowth, whereas inhibition of IGF1 attenuated D-4F- or IGF1-induced axonal growth. D-4F treatment did not increase OPC proliferation but significantly decreased OPC death. These data suggested that the increase of IGF1 may contribute to D-4F treatment–induced neurite and axonal outgrowth after stroke in mice.

Limitations

(1) We did not test whether D-4F improves functional outcome in nonstroke animals; we only tested the D-4F treatment effect in young (3- to 4-month-old) male stroke mice because female mice are a more complex preclinical model because of the hormonal changes associated with the estrus cycle. In addition, the therapeutic response to D-4F may differ between young and old subjects. Thus, the effects of D-4F on females and elderly animals, both male and female, warrant further investigation.

(2) We only evaluated the D-4F treatment effect on decreasing WM damage in the ischemic brain; whether D-4F regulates gray matter warrants investigation. (3) Given the experimental design of acute 2 h poststroke treatment, we cannot distinguish between WM-protective and WM-restorative effects. Clearly, D-4F did not decrease lesion volume, but it may protect WM.

(4) Previous studies have found that D-4F treatment significantly decreases brain arteriole inflammation and cognitive impairment. However, the scrambled D-4F treatment did not induce the beneficial effects.\textsuperscript{23} The scrambled D-4F control was not used in this study; therefore, our current findings have not yet been demonstrated to be specific to D-4F.

In summary, we are the first to report that D-4F treatment initiated 2 h after stroke significantly decreased neuroinflammation and WM damage and improved functional outcome 7 days after MCAo. IGF1 may mediate D-4F treatment–induced neurite/axonal outgrowth.

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Disclosures

None.

References


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