Deficiency of Brain ATP-Binding Cassette Transporter A-1 Exacerbates Blood–Brain Barrier and White Matter Damage After Stroke

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Background and Purpose—The ATP-binding cassette transporter A-1 (ABCA1) gene is a key target of the transcription factors liver X receptors. Liver X receptor activation has anti-inflammatory and neuroprotective effects in animal ischemic stroke models. Here, we tested the hypothesis that brain ABCA1 reduces blood–brain barrier (BBB) and white matter (WM) impairment in the ischemic brain after stroke.

Methods—Adult brain-specific ABCA1–deficient (ABCA1−/−B) and floxed-control (ABCA1 fl/fl) mice were subjected to permanent distal middle cerebral artery occlusion and were euthanized 7 days after distal middle cerebral artery occlusion. Functional outcome, infarct volume, BBB leakage, and WM damage were analyzed.

Results—Compared with ABCA1 fl/fl mice, ABCA1−/−B mice showed marginally (P=0.052) increased lesion volume but significantly increased BBB leakage and WM damage in the ischemic brain and more severe neurological deficits. Brain ABCA1–deficient mice exhibited increased the level of matrix metalloproteinase-9 and reduced the level of insulin-like growth factor 1 in the ischemic brain. BBB leakage was inversely correlated (r=−0.073; P<0.05) with aquaporin-4 expression. Reduction of insulin-like growth factor 1 and aquaporin-4, but upregulation of matrix metalloproteinase-9 expression were also found in the primary astrocyte cultures derived from ABCA1−/−B mice. Cultured primary cortical neurons derived from C57BL/6 wild-type mice with ABCA1−/−B astrocyte–conditioned medium exhibited decreased neurite outgrowth compared with culture with ABCA1 fl/fl astrocyte–conditioned medium. ABCA1−/−B primary cortical neurons show significantly decreased neurite outgrowth, which was attenuated by insulin-like growth factor 1 treatment.

Conclusions—We demonstrate that brain ABCA1 deficiency increases BBB leakage, WM/axonal damage, and functional deficits after stroke. Concomitant reduction of insulin-like growth factor 1 and upregulation of matrix metalloproteinase-9 may contribute to brain ABCA1 deficiency–induced BBB and WM/axonal damage in the ischemic brain. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.007145.)

Key Words: aquaporin 4 • ATP binding cassette transporter A-1 • blood–brain barrier • insulin-like growth factor binding protein 1 • stroke • white matter

The ATP-binding cassette subfamily member A-1 (ABCA1) regulates the efflux of cellular cholesterol and phospholipids to form apolipoprotein (apoE) lipoprotein particles and plays a critical role in high-density lipoprotein (HDL) cholesterol homeostasis and metabolism in the central nervous system.1–3 ABCA1 gene expression is regulated by liver X receptors, transcriptional factors involved in lipid metabolisms and inflammation.4,5 Activation of liver X receptors using synthetic liver X receptor agonists, GW3965, decreases neuroinflammation, promotes synaptic plasticity and axonal regeneration, and improves neurofunctional outcome after cerebral ischemia.6,7 ABCA1 has anti-inflammatory effects in both brain and the peripheral blood system, which implies its neuroprotective potential.8 Compared with wild-type mice, mice lacking brain ABCA1 exhibit fewer synapses and synaptic vesicle changes in neurons and increased neuroinflammation in the brain.3,5

The blood–brain barrier (BBB) is composed of brain capillary endothelial cells connected by tight junctions and pericytes that share common basement membrane extracellular matrix proteins that form the perivascular space. Vascular cells, including smooth muscle cells, brain capillary endothelial cells and pericytes, neural cells, ie, neurons, astrocytes, and oligodendrocytes, and microglia form the neurovascular unit which is the functional unit of the BBB.9,10 Cells forming the neurovascular unit express ABCA1 that plays an important role in maintaining BBB integrity.
role in the maintenance of BBB and neurovascular unit stability. However, the effects of ABCA1 on stroke-induced BBB and white matter (WM) damage have not been investigated.

Insulin-like growth factor 1 (IGF1) is not only involved in brain growth, development, and myelination but also in neurogenesis and oligodendrogenesis. IGF1 has neuroprotective properties and attenuates stroke-induced BBB damage concomitantly with rapid immunosuppression and sustained anti-inflammatory in brain. IGF1 decreases cholesterol efflux via ABCA1 and scavenger receptor class B type I expression. Matrix metalloproteinase-9 (MMP9) is a major contributing factor to BBB leakage after stroke. Whether MMP9 and IGF1 mediate brain ABCA1 deficiency–induced BBB and WM damages after stroke has not been investigated. In this study, we tested the effects and underlying mechanisms of brain ABCA1 deficiency in the regulation of BBB, WM and axonal damages, and functional outcome after stroke.

Methods

Animal Model and Experimental Group
Adult (2–3 months) male brain ABCA1 knockout (ABCA1-floxed x nestin-Cre positive, ABCA1(−/−)) and control (ABCA1-floxed, ABCA1(+/+)) mice were generated using the Cre/loxP recombination system. Mice were subjected to permanent distal middle cerebral artery occlusion (dMCAo) and were euthanized 7 days after dMCAo for neurological functional evaluation, immunostaining (n=9 in ABCA1(−/−) mice; n=11 in ABCA1(+/+) mice), western blot, and real-time quantitative polymerase chain reaction assays (n=4 per group). Sham-operated mice (n=6 per group) underwent the same surgical procedure without ligation of dMCAo.

Functional Test
The adhesive removal test, a sensitive test of somatosensory function in mice for dMCAo model, was performed before dMCAo and at 1, 3, and 7 days after dMCAo by an investigator who was blinded to the experimental groups, as previously described.

Histological and Immunohistochemical Assessment and Lesion Volume Measurement
Animal brains were fixed and 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 for the calculation of lesion volumes. Every 10th coronal section was cut from the center of the lesion (bregma −1 mm to +1 mm), and a total 5 of sections were used for immunostaining. Histochemical double staining for Bielschowsky silver (bregma −1 mm to +1 mm), and a total 5 of sections were used for immunostaining, and the percentage of BS+/LFB- area in the IBZ of the corpus callosum were analyzed at ×40 magnification with a Micro Computer Imaging Device imaging analysis system (Imaging Research).

Immunostaining Quantization
Images were acquired from 5 slides from each brain, with each slide containing 4 field views from the ischemic core, ischemic boundary zone (IBZ) of the cortex, or the corpus callosum, respectively (Figure 1A). The area that stained positive for albumin in the ischemic core area of cortex and for AQP4 in the IBZ of cortex, the number of PDGFRe+ and APC+C cells in the IBZ of cortex or corpus callosum, and the percentage of BS+/LFB- area in the IBZ of the corpus callosum were analyzed at ×40 magnification with a Micro Computer Imaging Device imaging analysis system (Imaging Research).

Primary Brain Astrocyte Cultures
The pregnant ABCA1(−/−) mice were euthanized, and E15 embryos were harvested. Genotyping was performed to identify ABCA1(+/−) (n=7) or ABCA1(−/−) (n=6) embryos. Primary astrocytes were isolated and cultured, as previously described. Passage 2 astrocytes were used and cultured with DMEM medium with t-glutamine without d-glucose and sodium pyruvate (Gibco) followed by 3 hours of oxygen–glucose deprivation (OGD). The astrocyte-conditioned medium was collected and stored at −80°C for primary cortical neuron (PCN) culture use. The astrocytes were used for western blotting and real-time quantitative polymerase chain reaction assay.

PCN Culture and Neurite-Outgrowth Measurements
First, we tested whether ABCA1 knockdown in astrocyte regulates PCN neurite outgrowth; PCN cultures with astrocyte-conditioned medium derived from ABCA1(−/−) and ABCA1(+/+) mice were used. PCNs were isolated from E15 embryos from a C57BL/6 wild-type mouse (Jackson Laboratory, n=8). On day in vitro 3, the PCNs were subjected to 3-hour OGD for hypoxic ischemia, and then treated with conditioned medium derived from ABCA1(−/−) or ABCA1(+/+) astrocytes for 24 hours (n=6 wells per group).
Second, we tested whether reduced ABCA1 in neurons regulates neurite outgrowth and whether IGF1 mediates the neurite outgrowth. PCN cultures were isolated from E15 embryos of ABCA1−B/−B (n=7) and ABCA1fl/fl (n=6). After 3-hour OGD, the experimental groups include (1) ABCA1−B/−B-PCNs, (2) ABCA1−B/−B-PCNs, (3) ABCA1−B/−B-PCNs treated with IGF1 (recombinant mouse IGF1 protein, 100 ng/mL; Abcam, Cat number ab9861, n=6 wells per group) for 24 hours. PCN cultures were immunostained for neuronal class III β-tubulin (a phenotypic marker of neural cells, 1:1000; Covance) with Cy3. Neuronal class III β-tubulin–positive cells and neurites were photographed at ×10 magnification using a fluorescent microscope. The average neurite length of the 20 longest neurites in each well (6 wells per group) was measured using the Micro Computer Imaging Device analysis system.

Real-Time Quantitative Polymerase Chain Reaction
The ipsilateral brain and the homologous areas from the sham brain (Figure 1A) and the harvested astrocyte cultures were used for real-time quantitative polymerase chain reaction. The following primers were designed using Primer Express software (ABI). GAPDH: AGAACATCATCCCTGCA(TCC (FWD) and CACATTTGGCTCCGGTATAAT (REV), IGF1: TGGATGCTCTTCAGTTCGTG (FWD) and TGGTAGATGGGGGCTGATAC (REV), MMP9: ATCTCTTCTTAGA-CTGGGAGGAGG (FWD) and AGCTGATTGACTAAAGTAGCTGGA (REV), and AQP4: CCGTTTCATGGAACCTCCTACT (FWD) and CATGCTGCTCGGTTAAT (REV).

Western Blotting
Specific proteins were visualized using Luminal Reagent (Santa Cruz). Anti-AQP4 (1:1000, Abcam), anti-IGF1 (1:1000, Abcam), anti-MMP9 (1:500, Santa Cruz). Anti-AQP4 (1:1000, Abcam), anti-IGF1 (1:1000, Abcam), and anti-β-tubulin (1:1000, Abcam) were used, as previously described.20

Statistical Analysis
Differences in the functional outcome and lesion volume were analyzed using Student t test. The percentage of albumin+, AQP4+, BS+/LFB+-area and APC+ cell numbers, protein and mRNA expression were analyzed using 2-factor ANOVA followed by post-hoc Bonferroni test. One-way ANOVA and least significant difference test were performed for neurite outgrowth. Correlation between the percentage of AQP4+ and albumin+ areas was tested by Pearson correlation coefficients.

Results
Brain ABCA1 Deficiency Worsens Functional Outcome After Stroke
There was a marginal increase in the lesion volume (P=0.052; Figure 1B) and a significant increase in neurological deficits at 1, 3, and 7 days after dMCAo in ABCA1−B/−B mice compared with ABCA1fl/fl mice (P<0.05; Figure 1C).

Brain ABCA1 Deficiency Increases BBB Dysfunction After Stroke
To test whether brain ABCA1 deficiency regulates BBB leakage after stroke, expression of albumin and AQP4 in the ischemic brain was measured. There was no albumin infiltration, evident in the nonstroke brains either in ABCA1−B/−B or in ABCA1fl/fl mice receiving sham surgery. However, albumin infiltration was observed near the ischemic core in both ABCA1−B/−B and ABCA1fl/fl mice. Albumin density was significantly increased in ABCA1−B/−B mice (P<0.05; n=11) compared with ABCA1fl/fl mice (n=9) 7 days after stroke (Figure 2A).

AQP4 protein expression was significantly decreased in both the sham brains and in the IBZ of the cortex in the ABCA1−B/−B mice compared with ABCA1fl/fl mice after stroke measured by immunostaining (Figure 2B) and western blot (Figure 2C; P<0.05) 7 days after dMCAo. In addition, the density of AQP4 was inversely correlated with the amount of albumin accumulation in the IBZ (Figure 2D; r =−0.73; P<0.05).

Brain ABCA1 Deficiency Increases Axonal and WM Damages in the Ischemic Brain After Stroke
WM is composed of bundles of myelinated axons, and oligodendrocytes are the only myelin-forming cells in the central nervous system and maintain long-term axonal integrity.19,22 To test whether brain ABCA1 deficiency regulates axon and WM damage after stroke, the density of BS+/LFB+ and the number of APC+ cells in the IBZ of corpus callosum were measured. There was no significant difference in BS+/LFB+ density and APC+ cell numbers in the sham brains between ABCA1−B/−B and ABCA1fl/fl mice. However, the BS+/LFB+ density and APC+ cell numbers were significantly decreased in the IBZ of the corpus callosum in ABCA1−B/−B mice (n=11) compared with the ABCA1fl/fl mice (n=9) after stroke (Figure 3A and 3B; P<0.05).

In adult animals, OPCs are present in the brain parenchyma after stroke and are generated in the subventricular zone of the lateral ventricle where they proliferate, migrate, and differentiate into new oligodendrocytes.10,22 To test whether brain ABCA1 deletion decreases OPC numbers, the number of PDGFβRα+ OPCs was also measured. We found that the number of PDGFβRα+ OPCs in the IBZ of the corpus callosum and cortex significantly increased in ABCA1fl/fl mice after stroke compared with sham brain. In addition, compared with the ABCA1fl/fl mice, ABCA1−B/−B mice with stroke exhibited significant decreases in PDGFβRα+ OPC numbers in the IBZ of the corpus callosum and cortex, which indicates that ABCA1 may regulate OPC generation after stroke (Figure 3C; P<0.05).

Brain ABCA1 Deficiency Increases MMP9 and Decreases IGF1 in the Ischemic Brain
MMP9 is a marker of neuroinflammation.16 IGF1 has anti-inflammatory properties.12,13 To elucidate the mechanisms responsible for the increased BBB leakage and WM damage and worse functional outcome in ABCA1−B/−B mice, expression of MMP9 and IGF1 was measured by western blot (Figure 4A) and real-time quantitative polymerase chain reaction (Figure 4B), respectively. The data show that MMP9 expression in the ischemic brains was significantly increased, whereas IGF1 expression was significantly decreased in both sham and ischemic brains in ABCA1−B/−B mice compared with ABCA1fl/fl mice (P<0.05; n=4 per group).

ABCA1−B/−B Decreases AQP4 and IGF1 and Increases MMP9 Expression in Primary Cultured Astrocytes
To investigate whether ABCA1 knockdown altered the expression of AQP4, IGF1, and MMP9 in astrocytes, primary brain
Astrocyte cultures were used. Both the protein and mRNA levels of MMP9 were increased, whereas AQP4 and IGF1 were decreased (Figure 5A and 5B) in the primary cultured ABCA1−/− astrocytes (n=7) compared with ABCA1fl/fl astrocytes (P<0.05; n=6) after OGD.

### Brain ABCA1 Deficiency Decreases Neurite Outgrowth, Whereas IGF Attenuates this Reduction

To investigate whether ABCA1 knockdown in both neurons and astrocytes regulates neurite outgrowth, the PCN and astrocyte cultures derived from E15 ABCA1−/− and ABCA1fl/fl embryos were used. Neurite outgrowth was significantly decreased in wild-type PCNs cultured with ABCA1−/− astrocyte-conditioned medium when compared with the ABCA1fl/fl astrocyte-conditioned medium treatment group (Figure 5C; P<0.05; n=6 per group).

To further elucidate the pathway responsible for the reduced neurite outgrowth in ABCA1 deficiency, IGF1 treatment was used. Neurite outgrowth was significantly decreased in ABCA1−/−-PCNs compared with ABCA1fl/fl-PCNs after OGD, whereas IGF1 (100 ng/mL) treatment significantly attenuated this reduction (Figure 5D; P<0.05; n=6 per group).

### Discussion

Stroke leads to BBB disruption and WM and axonal damages that are associated with long-term disability after stroke. Astrocytic endfeet are tightly attached to neurons on one side and to brain capillary endothelial cells and pericytes of the BBB on the other side, thereby connecting neurons and vascular cells, and play a decisive role in BBB maintenance.

In rodent brain, AQP4 is primarily expressed in astrocytic endfeet in contact with BBB and mediates water homeostasis. However, at a later phase post brain injury (7 days to 1 month), AQP4 may play a beneficial role and may inhibit activation of microglia and promote resolution of
edema. The upregulation of AQP4 causes increased water clearance from the tissue and decreased BBB disruption and neutrophil infiltration, as well as decreased proinflammatory cytokines. Using nestin-Cre system, ABCA1 was primarily knocked out from neural cells but not from brain capillary endothelial cells in ABCA1−/− mice. In this study, we found that brain ABCA1 deficiency significantly increased BBB leakage in the ischemic brain but decreased AQP4 expression in both the sham and the ischemic brain after stroke, and BBB leakage was inversely correlated with the AQP4 expression in the ischemic brain after stroke. In addition, a decrease of AQP4 expression was found in primary astrocyte cultures after OGD. These data indicated that brain ABCA1 deficiency decreases astrocytic endfeet protein expression, which may contribute to BBB dysfunction in the sham brain and increased BBB leakage after stroke. Pericytes also play a pivotal role in the formation and maintenance of the adult BBB. Pericytes project elongated, stellate-shaped finger-like processes that unsheath >70% of the abluminal side of the capillary wall, and astrocytes directly communicate with pericytes in addition to endothelial cells. Pericyte deficiency in the central nervous system leads to BBB breakdown and brain hypoperfusion, resulting in secondary neurodegenerative changes. Because pericytes also express nestin, therefore, we cannot exclude the possibility that ABCA1 is also deleted from pericytes, in addition to neural cells. Further studies on this issue are warranted.

BBB leakage post ischemic stroke might be, in part, explained by the increase vascular–associated MMP9 activity because tight junction proteins and basement membrane

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Figure 3. ABCA1−/− increases white matter damage and decreases oligodendrocytes and OPCs in the ischemic brain after distal middle cerebral artery occlusion (dMCAo). A, Bielschowsky silver (BS)-axon (black) and Luxol fast blue (LFB)-myelin (blue) double staining and quantitative data. B and C, Adenomatous polyposis coli (APC) and platelet-derived growth factor receptor α (PDGFRα) immunostaining and quantitative data. Scale bar, 40 μm (A) and 100 μm (B and C). IBZ indicates ischemic boundary zone.
extracellular matrix proteins are substrates for MMP9.\textsuperscript{9,16} MMP9 correlates with early-stage vascular dysfunction and BBB disruption, as well as WM damages\textsuperscript{26}; inhibition of MMP9 effectively reduces cerebral-vascular permeability and reduces brain infarction in mice after MCAo.\textsuperscript{30} Knockout of MMP9 significantly decreases BBB disruption and myelin basic protein reduction and prevents macrophage-induced axonal retraction after ischemia\textsuperscript{31}; MMP9 activity was significantly increased in the ischemic brain of type 2 diabetic mice which correlated with the increased WM damage after stroke.\textsuperscript{20} Neuroinflammation exacerbates BBB leakage in the ischemic brain, resulting in increased lesion volume and neurological deficits.\textsuperscript{29} ABCA1 is a potent anti-inflammatory factor. The systemic anti-inflammatory role of ABCA1 in the periphery has been studied using global ABCA1 knockout mice and macrophage-specific ABCA1 knockout mice.\textsuperscript{8} ABCA1\textsuperscript{−/−} mice also exhibit neuroinflammatory response, including increased nuclear factor $\kappa B$ and tumor necrosis factor-$\alpha$ gene expression in the brain.\textsuperscript{5} BBB dysfunction also promotes WM and axonal damages.\textsuperscript{15} Ischemia-induced WM injury is generated by selectively upregulating neuroinflammation and BBB damage, which may be associated with the increased infarction volume and neurological deficits.\textsuperscript{29} In this study, we found that brain ABCA1 deficiency increases MMP9 expression in both the ischemic brain and cultured astrocytes under OGD conditions, which may contribute to the induction of BBB leakage and WM damage in the ischemic brain. However, MMPs may be beneficial during delayed phases after stroke because MMPs modulate the brain extracellular matrix and may mediate and facilitate beneficial plasticity and remodeling during stroke recovery.\textsuperscript{32} MMP9 was upregulated in the peri-infarct cortex at 7 to 14 days after stroke and is colocalized with markers of neurovascular remodeling. Treatment with MMP inhibitors at 7 days after stroke suppresses neurovascular remodeling, increases ischemic brain injury, and impairs functional recovery at 14 days.\textsuperscript{32} Therefore, the long-term effects of MMP9 post stroke using this model warrants investigation.

IGF1 is not thought to easily cross the BBB. Local production from astrocytes and microglia is the main source of IGF1 in the brain.\textsuperscript{33} Astrocyte-specific overexpression of IGF1 protects hippocampal neurons and reduces behavioral deficits after traumatic brain injury.\textsuperscript{34} Exogenous administration of IGF1 into the cerebral lateral ventricle significantly reduces the BBB breakdown, brain edema formation, and

Figure 4. ABCA1\textsuperscript{−/−} increases matrix metalloproteinase-9 (MMP9) in the ischemic brain and decreases insulin-like growth factor 1 (IGF1) expression both in the sham and ischemic brains. A. Western blot (WB) and quantification data. B. Real-time quantitative polymerase chain reaction (RT-qPCR) data. dMCAo indicates distal middle cerebral artery occlusion.

Figure 5. ABCA1\textsuperscript{−/−} decreases aquaporin-4 (AQP4) and insulin-like growth factor 1 (IGF1) but increases matrix metalloproteinase-9 (MMP9) expression in the primary cultured astrocytes, decreases wild-type (WT) primary cortical neuron (PCN) neurite outgrowth cultured with the ABCA1\textsuperscript{−/−} astrocyte-conditioned medium compared with the ABCA1\textsuperscript{fl/fl} astrocyte-conditioned medium, which was attenuated by IGF1 treatment after oxygen–glucose deprivation. A and B. Western blot (WB; A) and real-time quantitative polymerase chain reaction (RT-qPCR) data (B) in astrocytes. C and D. Neurite-outgrowth data in WT PCNs (C) and ABCA1\textsuperscript{−/−} and ABCA1\textsuperscript{fl/fl}-PCNs (D).
cellular/tissue injuries. Administration of IGF1 within a few hours after brain injury is protective in both gray and WM and leads to improved somatic function. For example, intracerebroventricular infusion of IGF1 prevented the loss of oligodendrocytes and myelin basic protein density 4 days after ischemia reperfusion. In this study, brain ABCA1 deficiency decreased IGF1 expression in the sham and in the ischemic brain, as well as the hypoxic ischemic astrocyte cultures, which coexisted with BBB dysfunction and axonal impairment in the sham brain and increased BBB leakage and WM damage in the ischemic brain. These data indicate that brain ABCA1 deficiency–induced decrease in IGF1 may also contribute to the increased BBB leakage and WM damage after stroke. However, the mechanism underlying how ABCA1 regulates IGF1 expression is not clear and warrants investigation.

Conclusions

To our knowledge, this is the first study to demonstrate that brain ABCA1 deficiency increases BBB leakage, WM/axonal damage, and functional deficits after stroke. Concomitant reduction of IGF1 and elevation of MMP9 may contribute to brain ABCA1 deficiency–induced BBB and WM/axonal damage (Figure 6).

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Disclosures

None.

References


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