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

Xu Cui, MD, PhD; Michael Chopp, PhD; Alex Zacharek, MS; Joanna M. Karasinska, PhD; Yisheng Cui, MD; Ruizhuo Ning, MD; Yi Zhang, PhD; Yun Wang, MD, PhD; Jieli Chen, MD

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 apoE-containing lipoprotein particles and plays a critical role in high-density lipoprotein 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, T0901317 and 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.5,8 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 the BBB.

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From the Department of Neurology, Henry Ford Hospital, Detroit, MI (X.C., M.C., A.Z., Y.C., R.N., Y.Z., J.C.); Department of Physics, Oakland University, Rochester, MI (M.C.); Neural Protection and Regeneration section, Center for Neuropsychiatric Research, National Institute on Drug Abuse, NIH, Baltimore, MD (Y.W.); and Department of Medical Genetics, Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, British Columbia, Canada (J.M.K.).


Correspondence to Xu Cui, MD, PhD, Neurology Research, E&R Bldg, Room No. 3029, Henry Ford Hospital, 2799 West Grand Blvd, Detroit, MI 48202, E-mail tcui@neuro.hfh.edu or Jieli Chen, MD, Department of Neurology, Henry Ford Hospital, Detroit, MI 48202, E-mail jieli@neuro.hfh.edu

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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-inflammation 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 of 5 sections were used for immunostaining. Histochemical double staining for Bielschowsky silver (BS, an axon marker) and Luxol fast blue (LFB, a myelin marker) was used and cultured with DMEM medium with L-glutamine and sodium pyruvate (Gibco) followed by 3 hours of oxygen–glucose deprivation (OGD). The astrocyte-conditioned medium derived from ABCA1−/− and ABCA1fl/fl mice were used. The astrocytes were used and cultured with DMEM medium with l-glutamine without α-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.

**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 l-glutamine without α-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 ABCA1fl/fl astrocytes for 24 hours (n=6 wells per group).
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$^{-/-}$ mice compared with ABCA1$^{fl/fl}$ 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$^{fl/fl}$ or in ABCA1$^{+/+}$ mice receiving sham surgery. However, albumin infiltration was observed near the ischemic core in both ABCA1$^{fl/fl}$ and ABCA1$^{+/+}$ mice. Albumin density was significantly increased in ABCA1$^{-/-}$ mice ($P<0.05$; $n=11$) compared with ABCA1$^{+/+}$ 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$^{-/-}$ mice compared with ABCA1$^{+/+}$ 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.\textsuperscript{12,13} 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$^{-/-}$ and ABCA1$^{+/+}$ mice. However, the BS$^+$/LFB$^+$ density and APC$^+$ cell numbers were significantly decreased in the IBZ of the corpus callosum in ABCA1$^{-/-}$ mice (n=11) compared with the ABCA1$^{+/+}$ 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.\textsuperscript{10,22} To test whether brain ABCA1 deletion decreases OPC numbers, the number of PDGFR$\alpha^+$ OPCs was also measured. We found that the number of PDGFR$\alpha^+$ OPCs in the IBZ of the corpus callosum and cortex significantly increased in ABCA1$^{+/+}$ mice after stroke compared with sham brain. In addition, compared with the ABCA1$^{+/+}$ mice, ABCA1$^{-/-}$ mice with stroke exhibited significant decreases in PDGFR$\alpha^+$ 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.\textsuperscript{16} IGF1 has anti-inflammatory properties.\textsuperscript{12,11} To elucidate the mechanisms responsible for the increased BBB leakage and WM damage and worse functional outcome in ABCA1$^{-/+}$ 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$^{+/+}$ mice compared with ABCA1$^{+/+}$ mice (P<0.05; n=4 per group).

ABCA1$^{-/-}$ 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.9 In rodent brain, AQP4 is primarily expressed in astrocytic endfeet in contact with BBB and mediates water homeostasis.23 AQP4 may have biphasic effects in stroke injury and neuroprotection; for example, AQP4 is related to the early stages of cerebral edema and neuroinflammation within 2 to 4 days after ischemia.23,24 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...

**Figure 2.** ABCA1−/− increases blood–brain barrier leakage in the ischemic brain and decreases aquaporin-4 (AQP4) expression in both the sham and the ischemic brain after distal middle cerebral artery occlusion (dMCAo). A and B, Albumin and AQP4 immunostaining and quantitative data. C, AQP4 western blot (WB) and quantitative data. D, Correlation of AQP4 and albumin immunostaining. Scale bar, 40 μm (A and B). IBZ indicates ischemic boundary zone.
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 proteins.
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{29}; 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−/− mice also exhibit neuroinflammatory response, including increased nuclear factor κB and tumor necrosis factor-α 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; 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 6. Diagram shows the mechanism of brain ABCA1 deficiency–induced BBB leakage; WM/axon damage; BBB leakage and WM/axon damage contribute to the functional deficits.

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.


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