CXCR4 Antagonist AMD3100 Protects Blood–Brain Barrier Integrity and Reduces Inflammatory Response After Focal Ischemia in Mice

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Background and Purpose—Inflammatory response plays a critical role in propagating tissue damage after focal cerebral ischemia. CXCL12 is a key chemokine for leukocyte recruitment. However, the role of CXCL12 and its receptor CXCR4 in ischemia-induced inflammatory response is unclear. Here we use the pharmacological antagonist of CXCR4, AMD3100, to investigate the function of CXCL12/CXCR4 in regulating inflammatory response during acute ischemia.

Methods—Adult male CD-1 mice (n=184) underwent permanent suture middle cerebral artery occlusion (MCAO). AMD3100 was injected for 3 days (1 mg/kg/day) after MCAO. Brain water content, infarct volume, neurological score, and myeloperoxidase (MPO) expression and activity were examined at 24, 48, and 72 hours after MCAO. Proinflammatory cytokine RNA and protein levels in brain tissue were measured by RT-PCR and enzyme linked immunosorbent assay.

Results—Neurological score was greatly improved in AMD3100-treated mice compared with the control mice 3 days after MCAO (P<0.05). Brain edema–induced change of water content, IgG protein leakage, Evans blue extravasation, occludin, and ZO-1 expression in ipsilateral hemisphere were alleviated by acute treatment of AMD3100. MPO expression and activity revealed that AMD3100 profoundly reduced the number of MPO-positive cells in the ischemic region (P<0.05). It also attenuated proinflammatory cytokines including interleukin 6, tumor necrosis factor α, and interferon γ; their mRNA and protein levels changed accordingly compared with the controls (P<0.05).

Conclusions—CXCR4 antagonist AMD3100 significantly suppressed inflammatory response and reduced blood–brain barrier disruption after MCAO. AMD3100 attenuated ischemia-induced acute inflammation by suppressing leukocyte migration and infiltration, in addition to reducing proinflammatory cytokine expression in the ischemic region. (Stroke. 2013;44:190-197.)

Key Words: AMD3100 ▪ blood–brain barrier ▪ edema ▪ inflammation ▪ ischemia

Inflammatory response is involved in the pathogenesis of ischemic stroke. Acute inflammatory response is characterized by peripheral leukocytes, monocytes, and lymphocytes influx into the cerebral parenchyma and activation of endogenous microglia after focal cerebral ischemia. Inflammatory response in the brain begins with the movement of leukocytes across the blood–brain barrier (BBB), a multistep process that requires cells to pass through a perivascular space before entering the parenchyma. Thus, prevention of leukocyte adhesion, rolling, and penetration could reduce focal inflammatory response after ischemia.

Loss of BBB integrity is a hallmark of ischemic brain damage. Under normal conditions, the BBB acts very effectively to protect the brain from many chemicals and bacterial insults. The BBB becomes more permeable during ischemic injury. As a result, some drugs, viruses, and neutrophils/microphages are allowed to move across the BBB. In the acute phase of ischemic attack, leukocyte accumulation around the microvascular endothelial cells can greatly increase BBB permeability. However, the relationship between inflammatory response and BBB disruption is unclear.

Chemokines play a crucial role in leukocyte accumulation in ischemic lesions. The expression of multiple chemokines has been observed in models of experimental cerebral ischemia. CXCL12 is 1 of these chemokines. After cerebral ischemia, CXCL12 expression is upregulated in the ischemic perifocal region and is often accompanied by monocyte infiltration into the ischemic injured area. CXCL12 thus potentially mediates the neuroinflammatory pathogenesis. However, the detailed roles that CXCL12 and CXCR4 play in ischemia-induced inflammatory injury and their downstream impact on BBB integrity are unclear. AMD3100 is a well-documented...
specific antagonist of CXCR4 and widely applied as CXCL12/CXCR4 blocker. In the present study, we used a mouse MCAO model to investigate (1) whether AMD3100 treatment could reduce proinflammatory cytokine and chemokine activation as well as leukocytes accumulation; and (2) whether attenuation of ischemia-induced inflammatory response could prevent BBB disruption. We aimed to identify the role of AMD3100 in the pathogenesis of ischemia-induced inflammatory response.

Materials and Methods

Suture Middle Cerebral Artery Occlusion in Mice

Animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University, Shanghai, China. Adult male CD-1 mice (n=184) weighing 25–30 g were used in this study. Permanent middle cerebral artery occlusion (MCAO) was performed following previously described methods with minor modification. Briefly, mice were anesthetized with ketamine/xylazine (100mg/10mg/kg; Sigma, St. Louis, MO). Body temperature was maintained at 37±0.3°C using a heating pad (RWD Life Science, Shenzhen, China). After isolation of left common carotid artery and external and internal carotid artery, a silicone-coated 6-0 suture (Covidien, Mansfield, MA) was gently inserted from the external carotid artery stump to the internal carotid artery and stopped at the opening of the middle cerebral artery. The distance from the bifurcation of internal/external carotid artery to middle cerebral artery was 10±0.5 mm. Successful occlusion was verified by Laser Doppler Flowmetry (Moor Instruments, Devon, UK). Sham-operated mice underwent the same procedure except the suture was inserted along the internal carotid artery and then immediately withdrawn.

Administration of AMD3100

AMD3100 (Sigma), a specific CXCR4 antagonist, was injected intraperitoneally immediately after MCAO surgery for 3 consecutive days. AMD3100 was dissolved with normal saline to an injection concentration of 100 μg/ml. The dose of AMD3100 was 1 mg/kg/day, which is sufficient for blocking CXCR4 without causing stem cell mobilization. The same amount of normal saline was used in the control group.

Measurements of Brain Edema and Infarct Volume

The wet minus dry weight method was used for determining the water content of the ischemic brain at 24, 48, and 72 hours after AMD3100 treatment. Brains were quickly divided into ipsilateral cortex, ipsilateral striatum, contralateral cortex, and contralateral striatum and weighed immediately after euthanizing the animals. Brain samples were dried in an oven at 95°C for 24 hours. The dried samples were weighed again, and brain water content was calculated as: [wet tissue weight – dry tissue weight]/wet tissue weight]×100%. For measurement of brain infarction and edema using cresyl violet staining, brains were removed and frozen immediately in −40°C isopentane. A series of 20-µm-thick coronal sections from anterior commissure to hippocampus were cut, the first 1 selected in every 10 consecutive sections was mounted on slides. The distance between adjacent sections on the slides is 200 μm. A total of 18–25 sections were mounted. The entire set of brain sections with 200 μm space between each section was analyzed for infarct volume determination. The frozen sections were then stained with cresyl violet (Sigma). The ischemic area of each section was delineated by image analysis software (ImageJ, National Institutes of Health, Bethesda, MD). Infarct size was corrected for edema using the following formula: [1 – (total ipsilateral hemisphere – infarct)/total contralateral hemisphere]×100%. Infarct volume between 2 adjacent sections was calculated by formula: 1/3bh(S1+S2+√S1*S2).

Infarct volume was derived from the sum of all infarct volume between each adjacent section.

Neurological Deficiency Assessment

Neurological status was assessed by an investigator who was blinded to drug treatment and according to a 4-tiered grading system: 0, no observable deficits; 1, torso flexion to right; 2, spontaneous circling to right; 3, leaning/falling to right; 4, no spontaneous movement. This scoring system has been described previously and has been shown to correlate with infarct volume.

Western Blot Analysis

Brain tissue containing protein (40 μg) from ipsilateral or contralateral hemisphere were loaded onto 10% resolving gel for electrophoresis. Proteins were transblotted onto a nitrocellulose membrane (Whatman Inc., Florham Park, NJ) then immuno-probed with MPO primary antibody (R&D Systems, Minneapolis, MN; 1:200). The blots were incubated with HRP-conjugated secondary antibody and then reacted with an enhanced chemiluminescence substrate (Pierce, Rockford, IL). The result of chemiluminescence was recorded with an imaging system (Bio-Rad, Hercules, CA).

MPO Activity Assay

MPO activity assay method was described previously. Extracted brain protein (10 μl) from ipsilateral or contralateral hemisphere was added to 190 μl of assay solution, which consists of 2 mmol/L O-Dianisidin-dihydrochlorid (Sigma) dissolved in 180 μl of 50 mmol/L potassium phosphate buffer (pH=6) and 10 μl of 100 mmol/L H2O2. Changes in absorbance at 460 nm were measured over 10 minutes. MPO activity was expressed in units, where 1 unit represents the amount of enzyme degrading 1 μmol/L H2O2 per minute. Units of activity were normalized to 1 mg of protein.

Enzyme Linked Immunosorbent Assay Analysis

Tissue samples were taken from ischemic region of the brain. Protein levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon (IFN)-γ, IL-1β, IL-1α, and CD40L were quantified using an enzyme linked immunosorbent assay (ELISA) kit (Mosaic ELISA system, R&D systems, Minneapolis, MN) according to the manufacturer’s protocol. Samples were measured in duplicates. Readings from each sample were normalized for protein concentration.

Real-Time PCR

Total RNA from ischemic region was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and suspended in 40 μl of RNase-free water according to manufacturer’s protocol. RNA concentration was determined by a spectrophotometer (NanoDrop1000, Thermo, Wilmington, DE). The amplification was performed by a fast real-time PCR system (7900HT, ABI, Foster, CA) using SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China). A universal 2-step RT-PCR cycling protocol. Samples were measured in duplicates. Readings from each sample were normalized for protein concentration.

Immunohistochemistry

Brain sections (20 μm in thickness) were first incubated in 0.3% H2O2 in methanol for 30 minutes, and then incubated in PBS containing 0.1% Triton X-100 for 30 minutes at room temperature. Slides were blocked for 30 minutes in 10% (wt/vol) bovine serum albumin dissolved in PBS and incubated overnight at 4°C with primary antibodies. For ZO-1 or occludin staining, sections were rinsed with PBS and incubated with 2 secondary antibodies for 1 hour. The sections were examined using a confocal microscope (Leica, Solms, Germany).
Images were analyzed by Image Pro Plus 6.0 (Media Cybernetics, Bethesda, MD). The gap length is presented as percentage (%) of whole tight junction staining. For quantification, 4 vessels in perifactor regions were photographed. At least 4 serial sections spaced 200 μm were counted for each brain.

For MPO staining, sections were incubated for 30 minutes at biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and then incubated in Vectastain ABC solution for 30 minutes (Vector). After rinsing in PBS, the reaction product was visualized using DAB staining. Five fields in the lesion cortex, lesion basal ganglia, contralateral cortex, and contralateral basal ganglia were photographed in each section, and five serial sections spaced 200 μm were calculated for each mouse. MPO-positive cells were counted in each field by a person blinded to the identity of the photographs.

Brain immunolocalization of IgG was examined as reported. Briefly, brain slides were incubated with biotinylated universal antibody (Vector Laboratories) for 30 minutes, rinsed in PBS, and incubated in Vectastain ABC reagent for 30 minutes. After rinsing in PBS, the reaction product was visualized using DAB staining.

Evans Blue Extravasation
Two hours before the animals were euthanized, 4 mL/kg of 2% Evans blue (Sigma) in normal saline was injected into every animal. The animals were anesthetized and perfused with normal saline. For quantitative measurement of Evans blue leakage, the ipsilateral and contralateral hemisphere was removed and homogenized in 1 ml of trichloroacetic acid, then centrifuged at 21,000g for 20 minutes. Evans blue concentration was quantitatively determined by measuring the 610 nm absorbance of the supernatant.

Statistical Analysis
All results are expressed as mean±SEM. For comparison between 2 groups, statistical significance was determined through an unpaired Student t test. For comparison among multiple groups, statistical significance was evaluated with analysis of variance using Prism 4 software (GraphPad Software, San Diego, CA). A probability value of P<0.05 was considered significant.

Results
AMD3100 Attenuated Brain Edema and Improved Neurological Outcome
Infarct volume, examined by cresyl violet staining (Figure 1A), was decreased at 72 hours after MCAO in the AMD3100-treated mice as compared with the control mice (Figure 1B; P<0.001). The results of measured water content were consistent with infarct volume measurements, the AMD3100-treated group showing significantly reduced edema (Figure 1D; P<0.05). Ischemia-induced neurological deficits, assessed by neurological scores, were attenuated in AMD3100-treated mice at 72 hours after MCAO (Figure 1C; P<0.05). Brain edema, also examined by cresyl violet staining, was decreased at 48 and 72 hours after MCAO in the AMD3100-treated mice as compared with the control mice (data shown in Figure I in the online-only Data Supplement).

AMD3100 Reduced Leukocyte Infiltration
To determine whether CXCR4 antagonist AMD3100 could attenuate leukocyte infiltration into the ischemic region, we measured the number of MPO-positive cells and MPO activity in the ischemic hemisphere after MCAO. MPO activity is a good indicator of inflammation, and neutrophil accumulation can be quantified using the MPO activity assay. Results show that the number of MPO-positive cells was greatly increased in the ipsilateral hemisphere but not the contralateral hemisphere (Figure 2B). The number of MPO-positive cells was reduced in AMD3100-treated mice 48 and 72 hours after MCAO (Figure 2Ac; P<0.05). Western blot analysis showed that ischemia caused an increase in MPO levels in the ipsilateral hemisphere, whereas AMD3100 treatment attenuated such increase, albeit to a mild degree (Figure 2D). MPO activity in the ischemic hemisphere was
also reduced in AMD3100-treated mice 48 and 72 hours after MCAO (Figure 2C; \( P < 0.05 \)).

Brain Proinflammatory Cytokine mRNA and Protein Levels Were Reduced by AMD3100

Neuroinflammatory response was examined by measuring the mRNA and protein levels of inflammatory cytokines in the ischemic brain. Six cytokines were examined: TNF-\( \alpha \), IL-6, IFN-\( \gamma \), IL-1\( \beta \), IL-1\( \alpha \), and CD40L. We found that the mRNA levels of TNF-\( \alpha \) and IL-6 were reduced in AMD3100-treated mice at 24 hours after MCAO, whereas their proteins were reduced at 48 hours (Figures 3 and 4). IFN-\( \gamma \) expression was attenuated at 48 hours after MCAO (Figure 4C). It is notable that the mRNA and protein levels were not significantly changed for CD40L, IL-1\( \alpha \), and IL-1\( \beta \) in the AMD3100-treated group as compared with the control mice (Figure I in the online-only Data Supplement).

AMD3100 Protected the BBB

To evaluate BBB permeability after ischemic injury, BBB leakage was measured using IgG immunostaining and Evans blue extravasation. IgG leakage was detected at 3 days after...
MCAO in both AMD3100-treated and control mouse. We found that IgG leakage was significantly reduced in AMD-3100 treated mouse brain compared with the controls (Figure 5A). Evans blue extravasation was consistent with the IgG leakage (Figure 5B). To further investigate the effect of AMD3100 on tight junctions, we performed CD31/ZO-1 and CD31/occludin double staining. ZO-1 was continuously presented in the endothelial cell layer of microvessels in the sham brain (Figure 6A, arrow). Microvessel walls showed significant disruption with typical rearrangement and discontinuous staining along the margins of cell–cell contacts in the control mice after MCAO (Figure 6A, arrowheads). In contrast, the microvessel walls of the AMD3100-treated group presented much smoother and more continuous labeling, though there were still a few gaps (Figure 6A, arrowheads). The expression of occludin showed similar trend (Figure 6B). Quantitative analysis showed that total gap length increased in the control mice after MCAO, whereas such disruption was attenuated in the AMD3100-treated mice (Figure 6).

**Discussion**

AMD3100 significantly attenuated leukocyte accumulation and infiltration into the infarct perifocal region. It also effectively reduced brain edema and the level of proinflammatory cytokines in the brain tissue, protected BBB integrity, and improved neurological outcome. Collectively, these results show that AMD3100 can efficiently reduce brain injury in the acute phase of ischemic stroke, and that

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**Figure 3.** Inflammatory cytokine mRNA levels in the brain were reduced in AMD3100-treated mice. Bar graphs show the mRNA levels of TNF-α (A), IL-6 (B), IFN-γ (C), and IL-1β (D) in brain tissues at 24 to 72 hr after MCAO. Data are expressed as average copies per copy of GAPDH in 3 separate experiments. NS indicates normal saline-treated mice; AMD, AMD3100-treated mice. Data are mean±SEM, n=4 per group. *P<0.05, **P<0.025, AMD3100 vs. NS.

**Figure 4.** Inflammatory cytokine protein expression was reduced in AMD3100-treated mice. Bar graphs show the protein concentrations of TNF-α (A), IL-6 (B), IFN-γ (C), and IL-1β (D) in brain tissues at 24 to 72 hr after MCAO, measured by ELISA. NS indicates normal saline-treated mice; AMD, AMD3100-treated mice. Data are presented as mean±SEM, n=4 per group. *P<0.05, **P<0.025, AMD3100 vs. NS.
CXCL12 and CXCR4 might play important roles in the postischemic inflammatory response. The CXCL12/CXCR4 axis is one of the key signals for leukocyte trafficking. The early accumulation of neutrophils in the ischemic brain has been demonstrated by biochemical and histopathological studies. These neutrophils are found to adhere to the endothelium, initiating the infiltration process and enhancing the permeability of brain endothelium, which subsequently cause brain edema after ischemic attack. Recent studies indicated that CXCL12/CXCR4 enhances inflammatory infiltration of neutrophils during acute lung injury. Our results show that acute neutrophil infiltration was significantly reduced when AMD3100 was administered to ischemic stroke mice, indicating that CXCL12/CXCR4 is directly involved in neutrophil recruitment during the pathological development of acute inflammation after cerebral ischemia.

Figure 5. IgG leakage and Evans blue extravasation were attenuated in AMD3100-treated mice after MCAO. A, Photomicrographs show IgG staining in the ischemic perifocal area in AMD3100-treated and NS-treated mice after 72 hr of MCAO. Boxes in a and b are magnified to a, and b, whereas boxes in a, and b, are magnified to a, and b, respectively. Scale bar, 1 mm (a, b), 250 μm (a, b), and 50 μm (a, b), respectively. Arrows show IgG-positive staining, indicating leakage. B, Evans blue extravasation in brains after 72 hr of MCAO. Blue area shows extravasated Evans blue, indicating BBB disruption. Scale bar, 3 mm. Bar graph shows Evans blue extravasation index of ipsilateral/contralateral hemisphere. n=3–5 per group. *P<0.05, AMD3100 vs NS.

Figure 6. AMD3100 reverted rearrangement of ZO-1 and occludin. Confocal images show ZO-1 (A) and occludin (B) expression in sham, NS, and AMD3100-treated mice after 72 hr MCAO. Microvessels in the cortex of sham animal showed a continuous and linear labeling of ZO-1 or occludin along the whole vessel (arrows). Tight junction protein ZO-1 or occludin showed a discontinuous, less regular distribution in microvessels (arrowheads) in the ipsilateral cortex of NS-treated mice. Less disruption and fewer gaps (arrowheads) were detected in the ipsilateral cortex of AMD3100-treated mice. Scale bar, 10 μm. Bar graph shows the quantification of gap length. Data are mean±SEM, n=3 per group. *P<0.05, AMD3100 vs NS in ZO-1 study. **P<0.025, AMD3100 vs NS in occludin study.
Chemokine and cytokine signaling pathways have been found to cross-regulate each other’s expression levels during inflammation. CXCL12 increases the mRNA expression and secretion of IL-6 in cultured microglia. Our real-time PCR and ELISA data show that blocking CXCL12 signaling with AMD3100 reduced IL-6 mRNA levels at 24 hours after MCAO and subsequently reduced IL-6 plasma levels at 48 hours postischemia, suggesting that CXCL12 is an upstream regulator of IL-6 gene expression.

BBB disruption is a critical event in the progression of ischemic stroke. Hypoxia and inflammation lead to increased BBB permeability and disruption. The loss of tight junction proteins occludin and ZO-1 in the microvasculature has been shown to be likely mediated by cytokines such as monocyte chemoattractant protein-1, TNF-α, IL-1β, and IFN-γ. Administration of CXCR4 antagonist AMD3100 significantly reduced brain edema and rescued ischemia-induced ZO-1 disruption, suggesting that CXCL12/CXCR4 signaling also regulates BBB integrity through tight junction proteins.

Collectively, our study provides evidence that CXCR4 antagonist AMD3100 not only efficiently inhibited neutrophil recruitment during acute central nervous system inflammation but also facilitated the maintenance of BBB integrity after brain ischemic attack. Given that AMD3100 is mainly a CXCR4 antagonist, these results suggest that CXCL12/CXCR4 pathway is a potential target for drug development. However, it has been shown that many factors play a biphasic role at different stages of ischemic stroke. It should be noted that CXCL12/CXCR4 signaling play important roles in the recovery phase in recruiting stem cells that are important for neurovascular regeneration. Use of AMD3100 in the recovery phase may inhibit the recruitment of important stem cells, thus caution should be taken in choosing the treatment window.

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Disclosures

None.

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