Analgesine inhibits NF- κ B to regulate microglia polarization by TLR4 MyD88-dependent and MyD88-independent pathways in ischemic stroke

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Abstracts

Background: Microglia polarization plays an important role in poststroke recovery. Inhibition of proinflammatory (M1) polarization and promotion of anti-inflammatory (M2) polarization of microglia are potential therapeutic strategies for inflammation reduction and neuronal recovery after stroke. Analgesine (AGC), the extracts of *Vaccinia variola*-inoculated rabbit skin, is used to treat patients with chronic low back pain due to degenerative vertebral disorders. Here, we evaluated the neuroprotective effect of AGC in stroke and investigate anti-inflammatory mechanism of AGC on microglia-mediated neural damage.

Methods: Sprague-Dawley (SD) rats underwent 120 min of middle cerebral artery occlusion (MCAO) followed by reperfusion. We injected AGC intravenously into rats starting 3 h after the onset of MCAO. Then we investigated the effect of

AGC on neurological impairment, neuronal loss and inflammatory cytokines. For in vitro study, we examined the effect of AGC on microglial polarization in oxygen-glucose deprivation/reperfusion (OGD/R) or LPS/IFN- γ induced microglia cells and further investigated neuroprotective effect of AGC in microglia-mediated neural damage based on the direct or indirect co-culture systems. Finally, TLR4/Myd88/ NF- κ B pathway was detected in OGD/R-induced microglia cells with or without Myd88 siRNA transfection.

Results: AGC treatment reduced the neurological deficits and suppressed neuronal loss. In terms of inflammatory cytokines, AGC inhibited the release of pro-inflammatory cytokines and elevated the content of anti-inflammatory cytokines in vivo (SD rats) and in vitro (microglia). We further showed that AGC promoted M1 to M2 phenotypic transition of microglia in OGD/R or LPS/IFN- γ induced microglia cells. Based on the direct or indirect co-culture systems, we found AGC indirectly inhibits LPS/IFN- γ -induced neuronal damage by modulating microglial polarization. Moreover, AGC suppressed the nuclear translocation of the phosphorylation of NF- κ B p65 by inhibiting the TLR4/Myd88/TRAF6 but not TLR9 signaling. We also confirmed that AGC-regulated TLR4 inhibition partly dependent on Myd88 in a Myd88 depletion cell line.

Conclusion: Our findings provide a new understanding of AGC in neuroprotection by inhibiting M1 microglial polarization and promoting anti-inflammation by suppressing TLR4 MyD88-dependent and MyD88-independent pathways. Thus, AGC treatment may represent a novel approach in inflammation reduction or poststroke recovery.

Key words: Analgesine; microglial polarization; inflammation; cerebral ischemia; TLR4; MyD88; NF- κ B

Introduction

The vast majority of cerebral stroke cases are caused by ischemic stroke. The number of patients who have survived an ischemic stroke with a severe neurological deficit has now reached 33 million, and their number will increase to 77 million by 2030 [1]. The cerebral ischemia eventually leads to brain infarction. It typically occurs in the middle cerebral artery (MCA), which supplies the lateral convexity of the cerebral hemisphere and thereby the majority of the primary motor and sensory cortex, leading to contralateral hemiplegia with reduced sensation[2]. Only a minority of stroke patients are eligible for treatment, as delayed treatment can lead to worsened outcome. This worsened outcome at the experimental level has been attributed to an entity known as reperfusion injury (R/I) [3]. It results in activation of inflammatory processes that cause secondary tissue injury [4]. Anti-inflammatory Targets may be a promising therapeutic strategy to attenuate the progression of ischemic brain injury.

Microglia are macrophage-like cells located in the brain that play a biphasic role in the brain's inflammatory reactions to diseases of the central nervous system. After ischemic brain injury, microglia rapidly migrate toward the lesion site and exacerbate tissue injury by producing inflammatory cytokines and cytotoxic substances within hours. On the other hand, however, microglia also contribute to tissue repair and remodeling by clearing up debris. Differential polarization of microglia could likely explain the biphasic role of microglia in ischemia[5-6]. Ischemia-induced activated microglia can be polarized into a harmful phenotype, M1, and a protective phenotype, M2. M1 microglia produces pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF α), interleukin (IL)-1 β and IL-6. M2 microglia releases anti-inflammatory cytokines and neurotrophic factors [7-9].

Activated microglia expresses high levels of Toll-like receptors (TLRs) in the CNS and play a critical role in the modulation of inflammatory responses in several neuropathological conditions including ischemia/reperfusion injury [10-11]. Toll-like receptor 4 (TLR4), one of TLRs expressed on microglia surface, is involved in promoting microglia polarization [12-13]. TLR4 was upregulated in response to

ischemia-reperfusion in the brain suggesting that TLR4 may play a pivotal role in the pathogenesis of ischemic brain damage induced by middle cerebral artery occlusion (MCAO)[14]. Downstream signaling pathway of TLR4 activates myeloid differential protein-88 (MyD88) indirectly or directly trigger the translocation of nuclear factor kappa B (NF- κ B) into the nucleus and the expression of proinflammatory genes, involving the activation of the inflammasome [15]. Hence, inhibition of the TLR4/MyD88/NF- κ B signaling pathway to regulating microglia polarization may be an effective therapeutic strategy for ischemia stroke.

Analgesine (AGC), the extracts from rabbit skin inflamed by the vaccinia virus, contains inhibitors against the kallikrein-kinin system and effectively alleviates the chronic pain or allergic disease with little side effect for decades[16-18]. Recent study showed that AGC could also significantly reduce the infarct volume and ameliorated the neurologic deficits in animals after acute occlusion of the middle cerebral artery [19]. Although the neuroprotective effect on AGC has been studied, its underlying mechanism remains largely unknown. In this study, we aimed to investigate the effects of AGC on neurons and microglia after ischemic stroke injury. In addition, we studied the anti-inflammatory mechanism of AGC on microglia-mediated neural damage.

Materials and Methods

Drug

Analgesine (AGC®) is a natural bio-product obtained from rabbit skin extract through vaccinia virus inoculation inflammation as an antigen immune reaction. AGC (Lot. Y20180505) is provided by Vanworld Pharmaceutical (Rugao) Company.

Animals

Male Sprague–Dawley rats weighing from 260 to 300 g were purchased from Beijing Vitalriver Experimental Animal Co., Beijing, China and were housed at a temperature between 20 and 25 °C with a 12/12 h dark/light cycle and specific pathogen-free (SPF) conditions. During the entire experiment, the rats had free access to food and water. The animals were also weighed daily up to their sacrifice.

Middle cerebral artery occlusion and drug administration

The middle cerebral artery occlusion (MCAO) was induced by the intraluminal filament technique. Rats were anesthetized, then, a piece of suture thread (0.36mm in diameter) was inserted into the right internal carotid artery. Rats underwent 120 min of right MCAO by an intraluminal filament, then the filament was withdrawn, and the neck incision was closed with a silk suture. Rectal temperature was monitored throughout surgery and maintained between 37.0 and 37.5 °C with a heating pad during the surgical procedure. Upon awakening from anesthesia, rats were returned to their cages. In the sham-operated groups, the middle cerebral artery (MCA) was not occluded. All experimental procedures were carried out in accordance with the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation, and all efforts were made to minimize both the number of animals used and any suffering by the animals.

The animals were randomly divided into 6 groups after MCAO: sham group; AGC alone (20 U/kg; MCAO group; MCAO+AGC (10, 20, 40 U/kg) groups. AGC was dissolved in normal saline and injected intravenously via the tail vein once a day at the dose of 10, 20, 40 U/kg, starting 3 h after MCAO. The MCAO groups of ischemic rats and sham-operated rats received an equal volume of normal saline. After 24 h, the neurological functional test was evaluated by Longa 5-grade standard [19] (the higher the score, the severer the neurologic deficit) and then animals were sacrificed.

BV2 Cell Culture

The BV2 cell, a murine microglia cell line, was purchased from the National Infrastructure of Cell Line Resource. BV2 microglia were cultured in high-glucose Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% fetal bovine serum (FBS, from Gibco Invitrogen, Carlsbad, CA, United States), and 1% penicillin and streptomycin (Gibco Invitrogen, Carlsbad, CA, United States). The cell culture was maintained in a humidified atmosphere containing 5% CO₂ and 95% air, at a temperature of 37 °C.

When cells grew adherent and the cell bodies were branched, they were transferred into 6-well plates (10^5 cells per mL for Luminex and flow cytometry; Corning), 12-well plates (6×10^4 cells per mL for western blotting; Corning), 24-well plates (3×10^4 cells per mL for siRNA-transfection; Corning).

Primary neuron culture

Primary cultures with neurons were obtained from the brains of one-day-old C57 mouse. After the surface vessels and meninges were removed, the cortex grey matter was minced and incubated at 37°C for 7 min in D-Hanks containing 0.05% trypsin (Gibco Invitrogen, Carlsbad, CA, United States), and then dissociated mechanically into H-DMEM using a pipette. After the samples were filtered using 70-um mesh, the primary neurons were finally collected by centrifugation at 800 g for 5 min. Primary cortical neurons were incubated in 24-well plates with in DMEM-H supplemented with poly-L-lysine (0.1 mg/mL) for 4 h in a 5% CO₂ incubator at 37°C. The medium was then changed to neurobasal medium supplemented with 2% B27 and 0.5% glutamine. Cells were grown for 7 days (37°C, 5% CO₂) and culture medium was changed every 2 days.

Oxygen-glucose deprivation/reperfusion (OGD/R) treatment

After 80% confluence, the BV2 cells were washed thrice with PBS, then were treated with Na₂S₂O₄ (Sigma) at concentration of 10 mM in the DMEM medium (Gibco) for 1.5 h. The cultures were then returned to DMEM-H medium and incubated under normal conditions. AGC (0.25, 0.5, 1.0 U/mL) was given immediately after the OGD phase, acting through the processes of reoxygenation. After 3 h incubation, the cells were harvested.

LPS/IFN- γ -stimulated cells

BV2 cells were treated with LPS (100 ng/mL, Sigma) and IFN- γ (20 ng/mL, R&D system) in DMEM-H medium for 18 h, then cells were rinsed twice with cold PBS (pH 7.4). The cells were cultured with H-DMEM medium containing 2% FBS followed by treatment with or without AGC (0.25, 0.5, 1.0 U/mL) for 24 h.

Transfection of Myd88 Small Interfering RNA (Myd88 siRNA)

BV2 cells were transferred into 12-well plates (4×10^4 cells per mL, Corning). 0.18 μ mol MyD88-siRNA (Thermo) was transfected into BV2 cells for 43.5 h using Endo Fectin-Max Transfection Reagent (GeneCopoeia, USA) according to the manufacture's protocol. The transfection solution was removed and cells were rinsed with PBS and then replaced with H-DMEM medium containing 10% FBS 24 h after siRNA transfection. 15 h later, the BV2 cells were treated with OGD/R or AGC.

Mouse Cytokine and Chemokine detection by Inflammation Panel Luminex Kit

The concentrations of inflammatory cytokines in serum of cell supernatants were determined using Luminex technology with all samples tested in duplicate on a 96 well plate. The panel contained selected proinflammatory, inhibitory and chemotactic soluble cytokines (Eotaxon, GM-CSF, IFN- γ , IL-1 β , IL-6, IL-9, IL-12P40, IL-12P70, IL-17 α , MIP-1 β , TNF- α , IL-4, IL-10). Using the sigmoidal standard curve from the Millipore Analyst 5.1, a regression curve was extrapolated from the raw data individually for each cytokine.

Flow Cytometry

The culture supernatant was discarded, and the cells were washed gently with 1mL of PBS. Then, the adherent cells were digested and were centrifuged (2000g, 5min, 4°C). We washed the cells twice with PBS, and resuspended them at 1×10^6 cells per mL. To label the cells, we added FITC anti-mouse CD16/32 monoclonal antibody, PE anti-mouse CD206 monoclonal antibody for 15 min at room temperature in the dark, washed the cells twice with PBS, and resuspended them in 300 μ L PBS. The cell surface expression of CD16/32, CD206 was detected using a FACS Calibur flow cytometer (BD FACSCalibur), and the data were analyzed using Flowjo software.

Establishment of co-culture systems by transwell and direct contact methods

Primary neurons were seeded at a density of 3×10^4 cells per mL in poly-L-lysine (0.1 mg/mL)-coated 24-well plates (Corning) and incubated for 7 days.

BV2 cells were treated with LPS/IFN- γ and/or AGC, then the supernatant was discarded. The BV2 cells were carefully placed in the upper wells of transwell plates and co-cultured with primary neurons in the bottom wells.

According to the direct contact co-culture methods, primary neurons were seeded at a density of 3×10^4 cells per mL on poly-L-lysine (0.1 mg/mL)-coated 24-well culture and incubated for 7 days. BV2 cells treated with LPS/IFN- γ and/or AGC along with primary neurons were mixed at a density of 5×10^3 cells per mL for 24 h (37 °C, 95% air, 5% CO₂) in a fully humidified incubator.

Western blotting

A 100:2:1 mixture of 100 uL of ice-cold cell lysis buffer, cocktail, protease inhibitor (PMSF) was added to the cell culture, then cells were incubated on ice for 15min. Lysates were centrifuged at 12,000 g for 30 min at 4°C, and the supernatants were collected for analysis. A 5 × sodium dodecyl sulfate loading buffer was added, before incubating at 95°C for 5min. Then the samples were loaded at 20ug protein/lane on 10% acrylamide gels and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis for about 1.5h at 80mV and 120mV. Proteins were then transferred to a polycylnidene flouride membrane and blocking was made for 1h in a 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST). Samples were incubated at 4°C overnight with the primary antibodies, which are listed in Table 1. After washing three times with TBST, the samples were incubated for 1h at room temperature with a secondary antibody. Enhancement of the antibody reaction using an hypersensitive chemiluminescent reagent (ECL, Merck-Milipore) allowed for visualizing the protein. Protein bands were quantified using the Alpha View SA software (Protein Simple) and the band intensity was normalized to the band intensity of β -actin.

Immunohistochemistry

Mouse brains were fixed in 4% paraformaldehyde overnight, dehydrated in 30% sucrose and rapidly frozen in optimal cutting temperature compound (OCT)

embedding medium. The slices (thickness, 20 μm) were subsequently mounted on glass slides and stained for neuron as follows. Briefly, Brain slices were quenched to inactivate endogenous peroxidase for 30 minutes using 0.5% H_2O_2 ; then, the sections were incubated in 5% bovine serum albumin (sigma) for 20 min at 37 °C to block endogenous peroxidase activity. The sections were then incubated overnight at 4 °C with NeuN antibody at 4 °C overnight, followed by goat anti-rabbit or anti-mouse secondary antibodies (diluted 1:100) at room temperature for 30 minutes. Positive points were counted within fields of view of the same size (μm^2) under the microscope, and calculated using Image J software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

Immunofluorescence

After BV2 cells were treated with OGD/R, and the cell culture medium was discarded and washed three times with PBS. The 4% paraformaldehyde (Solarbio) was added for 20min at room temperature, after which the cells were again washed 3 $\times$ 5min with PBS. The membrane were disrupted with 0.5% Trion X-100 (PBS configuration) for 15min. After washing three times with PBS for 5min each, we added PBS containing 5% goat serum, and the plates were sealed at 37°C for 30min. The rabbit anti-NF- κB antibody (1:500) were added overnight at 4°C. After washing 3 $\times$ 5min with PBS, we added a fluorescently labeled goat anti-rabbit IgG secondary antibody (1:500) for 1h at 37°C. The cells were again washed 3 $\times$ 5min with PBS, and sealed with DAPI containing sealing agent. Then the samples observed under an Inverted fluorescence microscope (Olympus), and photographed.

After co-culture of BV2 cells and neurons, we observed the morphological changes of neurons. After treatment, the cells were washed 3 $\times$ 5min with PBS, and 4% paraformaldehyde (Solarbio) was added for 20min at room temperature, after which the cells were again washed 3 $\times$ 5min with PBS. The membrane were disrupted with 0.5% Trion X-100 (PBS configuration) for 15min. After washing three times with PBS for 5min each, we added PBS containing 5% goat serum, and the plates were sealed at 37°C for 30min. The rabbit anti-MAP2 antibody (1:500) were added

overnight at 4°C. After washing 3 × 5min with PBS, we added a fluorescently labeled goat anti-rabbit IgG secondary antibody (1:500) for 1h at 37°C. The cells were again washed 3 × 5min with PBS, and sealed with DAPI containing sealing agent. Then the samples observed under an Inverted fluorescence microscope (Olympus), and photographed.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5 software. One-way analysis of variance (ANOVA) was used for comparison between groups. When the P value in the student's ttest indicated statistical significance, the differences were assessed by the Dunnett's test. A value of $p < 0.05$ was considered to be statistically significant.

TABLE 1: Primary antibodies employed in this study.

Antibody	Type	Source
TLR4	Mono-	Santa Cruz
TLR9	Mono-	Abcam
Phospho-NF-κB p65 (Ser536)	Mono-	Cell Signaling
NF-κB	Mono-	Cell Signaling
TRAF6	Mono-	Abcam
MyD88	Poly-	Abcam
MAP-2	Poly-	Cell Signaling
CD16/32		Biolegend
CD206		Biolegend
goat anti-rabbit IgG		Invitrogen
goat anti-mouse IgG		Invitrogen
β-actin	Mono-	Cell Signaling

Results:

AGC improves neurological function of MCAO rats

The results showed that the intravenous injection of AGC starting at 3 h after MCAO significantly decreased Longa scores 24 h after MCAO, compared with the sham

group of ischemic rats ($P < 0.05$, Fig. 1), demonstrating that AGC was effective to improve neurological function in acute phase of cerebral ischemia.

AGC increases the number of neurons in the brain of MCAO rats

Since neuron-specific nuclear protein (NeuN) is the bona fide marker for mature neurons, we tested for its expression in different group. We found that the decreased expression of NeuN in the cortex of MCAO rats ($P < 0.05$, Fig. 2) were restored in the AGC-treatment ($P < 0.001$). This indicated that AGC treatment might have the neuroprotective effects on neurons damaged by ischemia stroke.

AGC modulates the level of inflammatory cytokines in the serum of MCAO rats

We detected the inflammatory cytokines in the serum of MCAO rats with inflammation panel luminex kit. Fig. 3 showed IL-4 was significantly decreased in the serum of rats 24 h after MCAO ($P < 0.05$), and IL-13 was also down-regulated. AGC could reverse the low level of IL-4 ($P < 0.05$) as well as IL-13 in the serum of MCAO rats. Monocyte chemoattractant protein-1 (MCP-1) and IL-18, the pro-inflammatory cytokines, had a trend to increase 24 h after MCAO. The release of MCP-1 was down-regulated by AGC ($P < 0.05$) along with IL-18.

AGC regulates OGD/R-induced inflammatory cytokines release in BV-2 Cells

To detect the effect of AGC on inflammatory cytokine release of microglia cells, inflammation panel luminex kit was used to measure the levels of GM-CSF, IFN- γ , IL-1 β , IL-6, IL-9, IL-12P40, IL-12P70, IL-17 α , MIP-1 β and TNF- α .

BV2 cells were subjected to oxygen and glucose deprivation (OGD) for 1.5 h followed by 1 h of reoxygenation (OGD/R), then cells were harvested with 0.25, 0.5, 1U/mL of AGC for 3 h. As shown in Fig. 4A, GM-CSF, IFN- γ , IL-1 β , IL-6, IL-9, IL-12P40, IL-12P70, IL-17 α , MIP-1 β and TNF- α was abnormally up-regulated in OGD/R-treated BV2 cells ($P < 0.001$). We found that AGC could reverse the high level of pro-inflammatory cytokine above ($P < 0.001$). The data in Fig. 4B showed the anti-inflammatory cytokines IL-4 and IL-10 were decreased after BV2 cells were subjected to OGD/R ($P < 0.001$), AGC treatment could increased the expression of

IL-4 and IL-10 ($P < 0.001$).

AGC switches microglia M1 to M2 transition

We used flow cytometry assay to detect the effect of AGC on the microglia M1 phenotype. M1 microglia were identified as viable CD16⁺CD32⁺ cells on flow cytometry. Fig. 5 showed the OGD/R-induced BV-2 cells showed a significant increased percentage of circulating CD16⁺CD32⁺ cells compared to the control group ($P < 0.05$). The CD16⁺CD32⁺ microglia were significantly lower in the OGD/R-induced BV-2 cells with AGC treatment ($P < 0.001$).

To further investigate whether AGC could switch microglia M1 to M2 transition

LPS/IFN- γ was used to induce microglia cells to M1 phenotype. M1 and M2 microglia were identified as viable CD16⁺CD32⁺ and CD206⁺ cells respectively on flow cytometry. In the present study shown in Fig. 6, the LPS/IFN- γ -induced BV-2 cells showed a significant increased percentage of circulating CD16⁺CD32⁺ cells and decreased percentage of circulating of CD206⁺ compared to the control group ($P < 0.01$), demonstrating microglia could transform to M1 state induced by LPS/IFN- γ . The CD16⁺CD32⁺ microglia were significantly lower and the CD206⁺ microglia were higher in LPS/IFN- γ -induced BV-2 cells with AGC treatment ($P < 0.01$).

AGC indirectly protects the morphology of neurons in microglia-mediated neural damage

To further explore possible effects of AGC on neuron survival after microglia-mediated insult, BV2 cells treated with LPS/IFN- γ and/or AGC (the supernatant was discarded), which were indirectly co-cultured with primary neurons (Fig. 7A) or directly co-cultured in transwell (Fig. 7C).

To assess changes in neuronal morphology after AGC treatment, MAP-2 protein was used to label neurons. MAP-2 is a neuron-specific cytoskeletal protein and plays a vital role in the formation and maintenance of dendrites during neuronal development [18]. In the activated BV2 cells (induction by LPS/IFN- γ) treated neurons, we found that MAP-2 (red) disappeared and the processes of cells were retracted, indicating that activated BV2 cells caused disintegration of the microtubular cytoskeleton. AGC

incubating with activated BV2 cells improved the structures of neurons and accompanied by notable increase in the number of neurons (Fig. 7B,D).

AGC deactivated the MyD88-dependent and independent pathways of TLR4

Western blots were performed to evaluate the influence of AGC on TLR4, TRAF6, MyD88, NF- κ B and phosphorylation of NF- κ B (p-NF- κ B). As shown in Figure 8A-F, OGD/R could increase the expression of TLR4, MyD88, TRAF6 and the p-NF- κ B/ NF- κ B ($P < 0.01$), while incubation with AGC reversed these changes ($P < 0.01$) except TLR9.

We further investigated effects of AGC on the activation of NF- κ B. The localization of NF- κ B protein was identified by western blotting and immunofluorescence. It demonstrated that OGD/R increased the expression of NF- κ B p65 subunit in nucleus while decreased its expression in cytosol ($P < 0.01$), indicating the nuclear translocation of NF- κ B p65 subunit. Treatment with AGC could suppress the activation of NF- κ B in OGD/R-induced BV-2 microglia cells ($P < 0.01$, Fig. 8H-K). In the immunohistochemistry experiment, we also found the expression of NF- κ B p65 (red) was increased by AGC treatment.

To further investigate whether CIG's inhibiting TLR4/ NF- κ B pathway dependent on Myd88, we transfected MyD88 siRNA into BV-2 microglia cells. Then MyD88 siRNA-transfected cells were subjected to OGD/R. In the MyD88 siRNA + OGD/R group, there was a trend toward an increase in the expression of p-NF- κ B/NF- κ B compared with OGD/R group. The expression of p-NF- κ B/NF- κ B presented an increasing trend in MyD88 siRNA + OGD/R group treated with AGC (Fig. 8L,M).

Discussion

The therapeutic options for ischemia stroke are limited. Analgesine (AGC) has been use in China and it has been inflamed by inoculation of *Vaccinia variola*. The clinical trials showed AGC protect against various somatic pain and neuropathic pain

[20–21]. In addition, AGC confers protection against neurological function damage induced by ischemia stroke in in vivo experimental conditions. However, the mechanism of the neuroprotective role of AGC remains unclear. In this study, we demonstrated that AGC displayed anti-inflammatory and inhibitory response to microglial activation to indirectly protect neurons after ischemic stroke injury. AGC inhibits ischemia-induced M1 microglial polarization and promotes anti-inflammatory effects by inhibiting TLR4/MyD88/NF- κ B.

Ischemia-reperfusion injury (I/R), produced by an initial interruption of organ blood flow and its subsequent restoration, contributes significantly to the pathophysiologies of stroke[22]. The ischemic stroke model induced by the middle cerebral artery occlusion and reperfusion (MCAO/R) has proven to be one of the most logical and effective animal stroke models[23]. So we evaluated the effects of AGC on ischemic stroke in the MCAO/R-induced rat model. We found AGC reduced the MCAO/R-induced neurological function impairment and neuronal death. I/R is a complex pathological process that begins with tissue anoxia and expands with the inflammatory response, which lead to neuronal death [24]. We also found AGC could inhibit the level of pro-inflammatory factors and increase the release of anti-inflammatory cytokines in the serum of MCAO/R-induced rat model. Microglia activation is the first step of inflammatory response after ischemic stroke [25–26]. We next investigated the anti-inflammatory mechanisms of ACG on microglial cells.

After stroke, the activated microglia become a double-edged sword, with distinct phenotypic changes to the deleterious M1 and neuroprotective M2 types [27–28]. M1-like microglia secrete pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-12, IL-18, and nitrogen monoxide (NO), and exacerbate inflammation and tissue injury. In contrast, M2-like microglia secrete anti-inflammatory cytokines, such as TGF- β , IL-4, IL-10, IL-13, and growth factors, suppress inflammation, and promote tissue recovery [29]. In the present study, results showed that AGC inhibited the level of pro-inflammatory cytokines and elevated the secretion anti-inflammatory cytokines in microglia cells after oxygen and glucose deprivation/reoxygenation (OGD/R). It demonstrated anti-inflammatory effect of AGC might be related to microglia

activation. The expression of M1 markers (CD16 and CD32) of microglia increased significantly after OGD/R, and AGC could down-regulated the number of CD16/32-positive cells, indicating AGC attenuated the microglia polarized to the M1 phenotype. The M1 phenotype microglia can be induced by lipopolysaccharide (LPS) or interferon-gamma (IFN- γ) with increased production of proinflammatory cytokines[30]. Treatment with AGC suppressed LPS/IFN- γ -mediated the expression of M1 marker CD16/32 and tended to elevate the CD206-positive M2 microglia number, demonstrating AGC could switch microglia M1 to M2 transition. Based on the above investigations, we believe that AGC resists inflammation by inducing microglial polarization to M2 phenotype. We also found that AGC inhibit LPS/IFN- γ -induced microglial damage to indirectly protect neuronal structure. However, the underlying mechanism how AGC regulates microglia polarization should be further studied.

Inhibition of nuclear factor-kappa B (NF- κ B) could promote M1 to M2 phenotypic conversion of microglia [31]. Post-translational modifications, particularly phosphorylation of the p65 sub-unit (NF- κ B/p65), are essential for cytoplasmic to nuclear localization of NF- κ B/p65 and initiation of transcription of downstream target genes[32]. In our current study, AGC significantly inhibited the phosphorylation of NF- κ B/p65 and its expression in the nuclear. It indicated AGC suppressed NF- κ B activity by attenuating translocation of its subunit from the cytosol to the nucleus.

NF- κ B is regulated by toll-like receptors (TLRs), which are a family of pattern recognition receptors that initiate signaling in innate and adaptive immune pathways[33]. TLRs family stimulates the activation of the downstream myeloid differentiation factor 88 (MyD88) and TNF receptor-associated factor 6 (TRAF6) to form complexes. Subsequently, MyD88-TRAF6 complex activate the downstream nuclear factor-kappa B (NF- κ B) [34-35]. By far, 11 human and 13 mouse TLRs have been identified and various evidences have confirmed that TLR4, TLR9 participate in the inflammatory reaction second to ischemia [36]. AGC could decreased the expression of TLR4 but not TLR9 induced by OGD/R. We further indicated MyD88 and TRAF6 were also down-regulated by AGC. These studies show that AGC

modulated microglial polarization through regulation of the TLR4/ MyD88/ NF- κ B.

TLR4 modulated NF- κ B through MyD88-dependent and MyD88-independent pathways[37]. In the present study, knockdown of MyD88 by siRNA abolished the effect of AGC on phosphorylation of NF- κ B p65. The above results showed AGC exhibited an inhibitory effect on TLR4 MyD88-dependent and MyD88-independent pathways.

Conclusion

The findings presented in this study provided novel mechanistic insights into how AGC mediated neuroinflammation after ischemic stroke. Herein, we demonstrated for the first time that AGC inhibited ischemia-induced M1 microglia polarization and promoting anti-inflammatory effects, which indirectly result in reduced ischemia-induced neuronal damage. In addition, AGC inhibited NF- κ B p65 to regulate microglia polarization by TLR4 MyD88-dependent and MyD88-independent pathways. Thereby, these findings provide a new understanding of the mechanism of AGC in ischemic stroke and provide a basis for the extending the use of AGC in clinical trials.

Abbreviations

AGC: Analgesine; MCAO/R: middle cerebral artery occlusion/ reperfusion; TNF α : Tumor necrosis factor alpha; IL: interleukin; TLRs: Toll-like receptors; MyD88: Myeloid differential protein-88; NF- κ B: The translocation of nuclear factor kappa B; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; TBST: Tris-buffered saline with 0.05% Tween-20; OGD/R: Oxygen and glucose deprivation/ reoxygenation; IFN- γ : Interferon-gamma; TRAF6: TNF receptor-associated factor 6

Declarations

Ethics approval and consent to participate

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Bioethics Committee of Xuanwu Hospital of Capital Medical University (authorization number: AEEI-2018-178).

Consent to publication

Not applicable.

Availability of data and material

The data and materials of this study are available from the corresponding authors for reasonable requests.

Competing interests

The authors have no competing of interest to declare.

Funding

This study was supported by Beijing Postdoctoral Research Foundation (2018-ZZ-105), China Postdoctoral Science Foundation (2019M650776), Capital Science and Technology Leading Talent Training Project (Z191100006119017), National Major Science and Technology Projects of China (No. 2015ZX09101-016).

Authors' contribution

Conception, design and manuscript writing: Cuicui Yang and Shili Gong; (II) Administrative support: Chaoying Hu, Lan Zhang; (III) Provision of study materials: Lin Li, Dongqing Zhang; (IV) Collection and assembly of data: Xiaoping Chen; (V) Data analysis and interpretation: Mingyang Wang; (VI) Final approval of manuscript: All authors

Acknowledgement

Not applicable.

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Legend

Fig 1. the effect of AGC on neurological function of MCAO rats.

Longa 5-grade standard (the higher the score, the severer the neurologic deficit) was measured at 24 h after MCAO. Data of Longa scores were expressed as mean $\pm$ SEM (n=8 per group). ### p <0.001 vs sham group; * p <0.05 vs MCAO group.

Sham: rats in sham operation group; Sham+AGC(20): sham-operated rats received Analgesine (20 U/kg); MCAO: rats underwent the middle cerebral artery occlusion (MCAO) operation; MCAO+ AGC(10, 20 or 40): MCAO rats received Analgesine (10, 20 or 40 U/kg).

Fig 2. the effect of AGC on the neurons in the brain of MCAO rats.

(A) Template of coronal brain section. The square represents the area which was selected to measure the number of NeuN+ cells. (B) Immunohistochemical staining of NeuN+ (neurons) in the cerebral cortex at 24 h after MCAO (20 $\times$ magnification). (C) Analysis for quantification of NeuN+ cells. Data were expressed as mean $\pm$ SEM (n=4 per group). # p <0.05 vs sham group; * p <0.05 vs MCAO group.

Sham: rats in sham operation group; Sham+AGC(20): sham-operated rats received Analgesine (20 U/kg); MCAO: rats underwent the middle cerebral artery occlusion (MCAO) operation; MCAO+ AGC(10, 20 or 40): MCAO rats received Analgesine (10, 20 or 40 U/kg).

Fig.3 Effects of AGC on the inflammatory response in the serum of MCAO rats

The release level of IL-4, IL-13, MCP-1 and IL-18 in the serum of rats 24 h after MCAO. Data were expressed as mean $\pm$ SEM (n=3-5 per group). # p <0.05 vs sham group; * p <0.05 vs MCAO group.

Sham: rats in sham operation group; Sham+AGC(20): sham-operated rats received Analgesine (20 U/kg); MCAO: rats underwent the middle cerebral artery occlusion (MCAO) operation; MCAO+ AGC(10, 20 or 40): MCAO rats received Analgesine (10, 20 or 40 U/kg).

Fig. 4 Effects of AGC on the inflammatory response in BV-2 cells after oxygen and glucose deprivation/reoxygenation (OGD/R).

The OGD/R cell model was established by oxygen glucose deprivation 1.5 h and reperfusion 1 h and then cell model was treated with AGC (0.25, 0.5, 1U/mL) for another 3 h. The protein levels of pro-inflammatory (A) and anti-inflammatory cytokines (B) of BV-2 cells were measured using inflammation panel luminex kit. The data are presented as the means $\pm$ SEM of four independent experiments (n = 4). ##P < 0.01, ###P < 0.001 vs. control group, *p < 0.05, **p < 0.01, ***p < 0.001 vs. OGD/R-treated group.

Ctrl (control): the normal BV2 cells; Ctrl+AGC-M: BV2 cells was treated with Analgesine (0.5 U/mL); OGD/R: BV2 cells were induced by oxygen and glucose deprivation/reoxygenation (OGD/R); OGD/R+AGC(L, M, H): OGD/R-induced BV2 cells was treated with Analgesine (0.25, 0.5 or 1 U/mL).

Fig. 5 Effect of AGC on activation of microglia M1 phenotypes after OGD/R

(A) M1 microglia cells in each group were identified by FITC anti-mouse CD16/32 antibody and analyzed by flow cytometry. (B) Relative percentage of the FITC-positive cells. The data are presented as the means $\pm$ SEM of four independent experiments (n = 4). #p < 0.05 vs. control group, **p < 0.01, ***p < 0.001 vs. OGD/R-treated group.

Ctrl (control): the normal BV2 cells; Ctrl+AGC-M: BV2 cells was treated with Analgesine (0.5 U/mL); OGD/R: BV2 cells were induced by oxygen and glucose deprivation/reoxygenation (OGD/R); OGD/R+AGC(L, M, H): OGD/R-induced BV2 cells was treated with Analgesine (0.25, 0.5 or 1 U/mL).

Fig. 6 Effect of AGC on activation of microglia M1/M2 phenotypes after LPS/ IFN- γ

BV2 cells were cultured with LPS/IFN- γ for 18 h and then treated with AGC for 24 h. (A,B) M1 or M2 microglia cells in each group were identified by FITC anti-mouse CD16/32 antibody of PE anti-mouse CD206 separately and then analyzed by flow cytometry. (C,D) Relative percentage of the FITC-positive and PE-positive cells. The

data are presented as the means $\pm$ SEM of four independent experiments ($n = 4$). $##p < 0.01$ vs. control group, $*p < 0.05$, $**p < 0.01$ vs. LPS/IFN- γ -treated group.

Ctrl (control): the normal BV2 cells; Ctrl+AGC-M: BV2 cells was treated with Analgicine (0.5 U/mL); LPS/IFN- γ : BV2 cells were induced by LPS/IFN- γ ; LPS/IFN- γ +AGC(L, M, H): LPS/IFN- γ -induced BV2 cells was treated with Analgicine (0.25, 0.5 or 1 U/mL).

Fig. 7 the effect of AGC on neurons by attenuating microglia-mediated inflammatory response

LPS/IFN- γ -induced BV2 cells (activated BV2) treated with or without AGC, which were then carefully indirectly or directly co-cultured with primary neurons. Antibodies to MAP-2 and confocal fluorescence microscopy were used to visualize the neuron morphology. (A) Schematic diagram of indirect co-culture with transwell. (B) The studies of AGC on neurons using an indirect transwell co-culture system. (C) Schematic diagram of direct co-culture. (D) The studies of AGC on neurons using an direct co-culture system. Scale bar = 100 μ m.

Fig. 8 AGC regulates TLR4/ MyD88/ NF- κ b pathway in BV2 cells after OGD/R.

Representative image of western blot (A) and the analysis of TLR4 (B), TLR4 (C), MyD88 (D), TRAF6 (E) and the p-NF- κ b/ NF- κ b (F). Measurement of p-NF- κ b in nuclear and cytosol by western blotting (H) and immunofluorescence (I). The expression of p-NF- κ b/ NF- κ b in the OGD/R-induced BV2 cells with or without Myd88 siRNA transfection detected by western blotting assay, AGC was incubated with the transfected cells for 24 h (J). The data are presented as mean $\pm$ SEM ($n = 4$). $#p < 0.05$, $##p < 0.01$ vs. OGD/R; $*p < 0.05$, $**p < 0.01$ vs. OGD/R.

Ctrl (control): the normal BV2 cells; Ctrl+AGC-M: BV2 cells was treated with Analgicine (0.5 U/mL); OGD/R: BV2 cells were induced by oxygen and glucose deprivation/reoxygenation (OGD/R); OGD/R+AGC (L, M, H): OGD/R-induced BV2 cells was treated with Analgicine (0.25, 0.5 or 1 U/mL).

Fig.9 Proposed scheme shows the mechanisms of AGC inhibiting microglia polarization to protect neurological function.