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Sinomenine inhibits microglia activation and attenuates brain injury in intracerebral hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) causes morbidity and mortality and commonly follows the reperfusion after an ischemic event. Microglial activation mediated cytokine and protease secretion contributes to brain injury in ICH. Previous studies have shown that sinomenine possesses potent immunoregulatory properties. However, little is known about its exact role in ICH. In the present study, to investigate the effect of sinomenine on microglial cells inflammation, we treated ICH-challenged BV2 microglial cells with sinomenine in vitro, and explored its neuroprotection role in intracerebral hemorrhage in vivo. Changes in inflammatory cytokines, such as TNF-α, IL-1β and IL-6, reactive oxygen species (ROS) and NF-κB activation NF-κB were observed. In addition, the neurological deficit and cerebral water content of ICH mice were studied. The results demonstrated that sinomenine could inhibit the release of these cytokines and attenuate ROS production in a dose-dependent manner, and reduce NF-κB activation. Furthermore, sinomenine markedly inhibited cerebral water content and neurological deficit. In conclusion, our findings suggest that sinomenine played the protective effects through inhibition of microglial inflammation, and the findings also provided a novel therapy to treat ICH induced brain injury.

1. Introduction

Intracerebral hemorrhage is a devastating type of stroke, accounting for 10–15% of all strokes (Huttner et al., 2008; Kuramatsu et al., 2010; Graffagnino et al., 2013). The 30-day mortality rate is 45% and most survivors are left with a neurological disability (Beslow et al., 2010; Leker et al., 2009; Christensen et al., 2009). Undoubtedly, much tissue damage and distortion occur at the time of hematoma formation, but secondary brain injury and edema formation may also be important and susceptible to treatment (Ducruet et al., 2009; Zuo et al., 2009; Sykora et al., 2009). Increasing evidence has shown that inflammation is the key contributor of ICH-induced secondary brain injury. Inflammation progresses in response to various stimuli produced after ICH.

Current experimental evidence demonstrates that microglia activation mediated inflammatory response contributes to secondary brain injury (Wang et al., 2013; Zhao et al., 2007; Szymanska et al., 2006). In addition, several mediators present in blood, such as thrombin and complement, are involved in the brain inflammation that occurs following central nervous system injury (Keep et al., 2005; Xu et al., 2013; Xi et al., 2002). However, there are currently no US Food and Drug Administration-approved antiinflammatory or cytoprotective agents for ICH.

Sinomenine has been used successfully for centuries in the treatment of patients with various rheumatoid diseases (Chen et al., 2011; Zhao et al., 2012; Kok et al., 2005). Many studies have investigated the potential immunosuppressive mechanisms of sinomenine, and results have shown that sinomenine significantly reduces the secretion of prostaglandin E3, leukotriene C4, nitricoxide and tumor necrosis factor-α (TNF-α) from activated macrophages, while inhibiting IL-8 and enhancing IL-6 secretion from peripheral blood monocytes (Liu et al., 1994).

Unfortunately, whether sinomenine has the immunoregulatory potential in ICH-induced inflammatory injury is still unclear. Therefore, in the study, we assessed the effect of sinomenine on the ICH-induced microglial inflammation and neurological deficit.

2. Materials and methods

Sinomenine (purity > 99%), a white crystalloid powder, was obtained from Zhengqing Pharmaceutical Group (Hunan, China).

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3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) containing L-arginine (200 mg/l)-fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco (Grand Island, NY, USA). Other chemicals were purchased from Sigma.

2.1. Cells and animals

The murine microglial cell line BV2 was purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific Hyclone, Logan, UT, USA), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The cell line was kept at 37 °C in a humidified atmosphere containing 5% CO_2. Male C57BL/6 mice (8–10 weeks) were obtained from the Animal Center of the Third Military Medical University and bred under specific pathogen-free conditions. Experiments were conducted in accordance with animal care guidelines approved by the Animal Ethics Committee of the Fujian Medical University.

2.2. Cell culture

BV2 microglia collected from culture flasks were seeded at a density of 3×10^5 cells/well onto 24-well tissue culture plates. One day after seeding, each culture well was fed with medium containing 0 (control) or 0.01, 0.1, 1 mM sinomenine (Xi'an High-tech Co., Xi'an, China) for 1 h, and then was stimulated with 10 μl erythrocyte lysis. After 3 days, the supernatants were removed and further analyzed for cytokine production with ELISA.

2.3. Effect of sinomenine on microglia viability

Alterations of microglia viability were determined by measuring the release of LDH using a kit purchased from Roche (No. 1644793; Grenzach-Wyhlen, Germany). Briefly, one day after seeding, each culture well was fed with medium containing 0 (control) or 0.01, 0.1, 1 mM sinomenine (Xi'an High-tech Co., Xi'an, China) for 1 h, the supernatant was collected to measure the release of LDH, and the amount of LDH in the supernatant was expressed as the percentage of total LDH released from lysed microglial cells.

2.4. Semi-quantitative measurement of intracellular ROS

Microglia collected from culture flasks were seeded at a density of 1×10^6 cells/ml onto glass bottom culture dishes. One day after seeding, each culture well was fed with medium containing 0 (control) or 0.1, 1 mM sinomenine, 1 h prior to the addition of 0 (control) or 10 μl erythrocyte lysis for 24 h. This was followed by addition of the ROS probe 2-, 7-dichlorodihydrofluorescein diacetate (H2DCF-DA, 5 μM/l) to the cells. The oxidation of the nonfluorescent H2DCF-DA by intracellular ROS results in the formation of the fluorescent compound 2-, 7-dichlorofluorescein (DCF). DCF mean fluorescence intensity (MFI) was monitored with a laser confocal scanning microscope (Model, TCS NT, Leica, Germany). The obtained data were analyzed using Zeiss LSM software and numerical presentation was conducted with Excel.

2.5. ELISA

BV2 microglial cells collected from culture flasks were seeded at a density of 3×10^5 cells/well onto 24-well tissue culture plates. One day after seeding, each culture well was fed with medium containing 0 (control) or 0.01, 0.1, 1 mM sinomenine (Xi'an High-tech Co, Xi'an, China) for 1 h, and then was stimulated with 10 μl erythrocyte lysis. After 3 days, the supernatants were removed, and IL-1β, IL-6, and TNF-α were measured by using ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance at 450 nm was determined using a microplate reader.

2.6. Determination of NF-κB activity by electrophoretic mobility shift assay

Nucleoprotein was extracted from brain tissue according to the manufacturer's instructions. Ten microgram of nucleoprotein of each sample was incubated with the reaction buffer at room temperature for 15 min, then P32-labeled oligonucleotide (5′-GGGGACTTTCC-3′; Life Technologies, Gaithersburg, MD, USA), which binds to NF-κB, was added to the reaction buffer and incubated for 15 min.After incubation for 20 min at 25 °C, the reaction mixture was subjected to 6% non-denaturing polyacrylamide gel electrophoresis. Autoradiography was performed at room temperature. Finally, the images were analyzed using an Bio-Rad Image Analyzer (CA, USA) and the results were expressed as optical density (OD).

2.7. Scratch assay

For scratch assay, 5×10^5 BV-2 cells were grown in 6-well plates as 80% confluent monolayers and were wounded with a sterile 100 μl pipette tip. Thereafter, the cells were stimulated with 10 μl erythrocyte lysis, 1 mM sinomenine, 10 μl erythrocyte lysis + 1 mM sinomenine, or control. Migration into the open scar was documented with microphotographs at different time points after wounding. The number of migrating cells was quantified by counting all cells within a 0.4 mm^2 region in the center of each scratch. A minimum of 5 individual cultures was used to calculate the mean migratory capacity of each cell culture condition.

2.8. Transwell migration assay

The Costar Transwell System (8-μm pore size polycarbonate membrane) was used to evaluate vertical cell migration. 1 Mio BV-2 cells in 1.5 ml serum-free medium were added to the upper well, and 2.6 ml serum-free medium was added to the lower chamber. To the lower chamber medium, 10 μl erythrocyte lysis, 1 mM sinomenine, 10 μl erythrocyte lysis + 1 mM sinomenine, or control were added. At the end of a 24-h incubation period, cells that had migrated to the lower surface were quantified by counting the migrated cells on the lower surface of the membrane using microscopy.

2.9. ICH mouse model

Mice were anesthetized and placed in a stereotaxic frame (Stoelting, USA). Approximately 25 μl of autologous blood was collected from the ventral tail artery or lateral tail veins. Then, the withdrawal site was disinfected, a 25 G needle was inserted into the vein and a capillary tube was used to collect the blood from the hub. The cannula was positioned over the entry point (EP) and introduced into the left striatum (coordinates: 0.2 mm anterior, 2.3 lateral and 3.5 mm ventral to the bregma). Firstly, 5 μl of blood was injected into the target point at a rate of 2 μl/min. For control groups, the injection should be performed using the abovementioned method, but the actual injection should be avoided. The remaining portion of blood was injected into the target site at a rate of 2 μl/min, 7 min later, and the needle was retained for 10 min. After pulling out the needle, the drilled hole was closed using bonewax. Sinomenine was given intraperitoneally at a dose of 20 mg/kg