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XQ-1H protects against ischemic stroke by regulating microglia polarization through PPARγ pathway in mice

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ABSTRACT

Cerebral ischemic and reperfusion injury often accompany with inflammation, and lead to severe neuronal damage, which further result in neurological disorders and memory disorders. In this study, we researched XQ-1H, a novel derivative of ginkgolide B, protecting against ischemic stroke in mice through regulation of microglia polarization. Middle cerebral artery occlusion (MCAO)/reperfusion in mice is applied to mimic ischemic stroke in vivo. Immediately after MCAO, mice are intragastric administrated with different dose (31 or 62 mg/kg) of XQ-1H for one or continuative three days. The in vivo experiments indicated that post-treatment with XQ-1H decreased cerebral infarction size, lessened brain edema, improved behavior and memory recover, inhibited pro-inflammatory and promoted anti-inflammatory cytokines expression and releasing in MCAO mice. Oxygen-glucose deprivation/reoxygenation (OGD/R) injury in BV-2 (microglia) cells is served in vitro. The in vitro findings revealed that incubation with XQ-1H protected against BV2 from OGD/R injury, regulated BV2 polarized from pro-inflammatory into anti-inflammatory phenotype, and promoted PPARγ mobilizing from nuclear to cytoplasm. In conclusion, the present study demonstrates that XQ-1H alleviated ischemic stroke by regulating balance of pro-/anti-inflammatory microglia polarization through PPARγ pathway both in vivo and in vitro, offering an alternative medication for stroke associated with inflammation.

1. Introduction

Ischemic stroke is a disease of high morbidity and mortality worldwide, and has negative impact on human health [1]. Cerebral ischemic and reperfusion injury often accompanied with inflammation, and lead to severe neuronal damage, which further result in neurological disorders and memory disorders [2]. Microglia derived macrophages are important cells in the immune system, which play crucial roles in central nervous system (CNS) repair and regeneration [3].

Microglia are mononuclear phagocytes located in the CNS [4], which are in the resting state under normal circumstances [5]. However, in the presence of damage factors, microglia can be activated into two states: “classical activation” (M1) and “alternative activation” (M2) [6]. The microglia classical activation secretes pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), contributing to the inflammatory response and leading to worse outcomes after cerebral ischemia [7]. Whereas alternative activation of microglia generates IL-10, IL-4, transform growth actor-β (TGF-β) and some of the cell repair factors, which relate to the resolution of neuroinflammation and anti-inflammation [8]. The imbalance of classical and alternative activation of microglia in the brain tissue directly leads to the occurrence of some central nervous system diseases [9]. Therefore, medicines that inhibit pro-inflammatory phenotypes or promote the transition to anti-inflammatory phenotypes after ischemic stroke are important for the treatment of ischemic stroke.

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated nuclear receptor with potent anti-inflammatory properties that modulates the immune inflammatory response [10]. In recent years, studies found that PPARγ and ischemia injury have close relations, and the effect of PPARγ on ischemic injury is considered mainly related to its regulating inflammatory response [11]. Activation of PPARγ signaling can reduce inflammation so that to play a protective role in CNS [12].

Ginkgolide is the main component of *Ginkgo biloba* extract preparation and has the strongest activity, in which Ginkgolide B (GB) has preventive and therapeutic effect of atherosclerosis and ischemic injury [13]. 10-O-(*N*,*N*-dimethylaminoethyl)-ginkgolide B methanesulfonate (XQ-1H), a novel derivative of ginkgolide B [14], exhibited strong antagonist of platelet activating factor. Previous studies have shown that XQ-1H has protective effects on ischemia reperfusion injury in rats by

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alleviating inflammatory mediators and neutrophils infiltration [15,16]. It was reported that XQ-1H contribute to improving ischemic stroke by extenuating the accumulation of inflammatory cells and alleviating blood brain barrier (BBB) disruption after ischemic stroke [17]. Nevertheless, the influence of XQ-1H on microglia polarization and stroke outcome in mice has not been reported.

In this study, we investigated the effects and mechanisms of XQ-1H on inflammatory cytokines release in mice of middle cerebral artery occlusion (MCAO)/reperfusion injury and BV-2 (microglia) cells subjected to oxygen-glucose deprivation/reoxygenation (OGD/R) injury. In vivo, we explored the effect of XQ-1H on cerebral infarction, brain edema, learning and memory function by observing pro-/anti-inflammatory phenotype and release of inflammatory cytokines. In vitro experiments further revealed the regulating effect and mechanism of XQ-1H on BV-2 cells through PPARγ pathway.

2. Materials and methods

2.1. Animals and treatment

Three hundred and twenty-five adult male C57BL/6 mice (20–25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were maintained 5 per cage in plastic bottomed cages containing wood shavings as bedding in a temperature-controlled room (22 ± 2 °C), with a 12 h light-dark cycle and a relative humidity of 60 ± 10%. Mice were allowed free access to food and water before the experiment, and were fasted for 12 h before surgery. Mice were randomly divided into 7 groups: sham, MCAO/R, XQ-1H (31 mg/kg), XQ-1H (62 mg/kg), Ginaton (30 mg/kg), GW9662 (1 mg/kg), GW9662 (1 mg/kg) + XQ-1H (62 mg/kg). For evaluation of infarct size, brain edema and neurological deficiency, n = 6 for each group. For Y-maze and rotarod test, n = 8 per group. For ELISA analysis, n = 4 per group. For immunofluorescence and real time PCR, n = 3 for each group. All animals were cared for in compliance with institutional guidelines of China Pharmaceutical University (Nanjing, China). All efforts were made to minimize animal suffering and reduce the number of animals used. Five mice died from anesthesia and ten died from intracranial hemorrhage, and the data from these mice were excluded in the analysis.

2.2. Drug administration

XQ-1H (Jiangsu Kefeiping Pharmaceutical Company Limited) was dissolved in saline. The mice were received different doses of XQ-1H (62 mg/kg and 31 mg/kg) by intragastric administration immediately after MCAO surgery, which with a volume of 0.1 ml/10 g and 3 times per day for 1 or 3 days [17]. The interval time of administration was 8 h. In vitro, the BV-2 cells were treated with XQ-1H at different concentrations of 1, 3 and 10 μM for 24 h before inducing OGD/R [15].

GW9662 (PPARγ inhibitor, Selleck Chemicals) was dissolved in DMSO and then was diluted with saline. The mice were injected at a dose of 1 mg/kg by intraperitoneal administration at 1 h before MCAO (0.1 ml/10 g) for one time. In vitro, GW9662 were incubated at a concentration of 1 μM for 24 h before OGD/R [18].

Ginaton (Dr. Willmar Schwabe GmbH) was dissolved in 0.5% sodium carboxymethylcellulose (CMC-Na) and was given for 3 times per day at a dose of 30 mg/kg. Mice were given Ginaton for 1 or 3 days after MCAO/R by intragastric administration immediately after MCAO with a volume of 0.1 ml/10 g and the interval time of administration was 8 h.

2.3. Cell culture and OGD/R injury

Murine BV-2 microglial cells were obtained from China Pharmaceutical University and cultured in DMEM/F12 1:1 high glucose (Hyclone Laboratories, Inc.) supplemented with 10% fetal bovine serum at 37 °C in humidified atmosphere containing 5% CO_2.

BV2 cells during logarithmic phase were adjusted to 1.6×10^5 cells/ml and seeded in cell plates. XQ-1H (1, 3 and 10 μM) or GW9662 (1 μM) was applied to the cells 24 h before OGD/R. Thereafter, the BV2 cells were maintained in serum/glucose-free DMEM and were introduced into an incubator containing 1% O_2/94% N_2/5% CO_2 at 37 °C for 3 h to establish conditions of OGD. Then the cells were returned to high-glucose medium under normoxic conditions with 95% air and 5% CO_2 as OGD/R [19]. The cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Beyotime Institute of Biotechnology) assay. 20 μl of MTT solution (5 mg/ml) was added per well. After 4 h of incubation, the supernatant was removed and 150 μl DMSO was added to solubilize the purple formazan product of the MTT reaction. Then the optical density (OD) value at 490 nm was measured by microplate reader and the cell viability was calculated by following formula:

$$\text{Cell viability} = (\text{OD value of administration group} - \text{OD value of solvent control group}) / (\text{OD value of sham group} - \text{OD value of solvent control group}) \times 100\%$$

2.4. Focal cerebral ischemia establishment

MCAO/reperfusion injury was served as ischemic stroke model in our experiment as described previously [20,21]. Mice were anesthetized by 3% chloral hydrate (300 mg/kg, i.p.) and fixed in the supine position. Briefly, a silicon-coated monofilament nylon suture (diameter about 0.16 mm, Beijing Cinontech Co. Ltd.) was inserted to occlude the origin of MCA. The filament was left for obstruction for 45 min and then withdrawn for the reperfusion. During the whole experiment, body temperature was maintained at 37 ± 0.5 °C. The sham group was induced by an identical operation without occlusion of the MCA. All other groups were performed MCAO/reperfusion surgery.

2.5. Neurological deficiency evaluation

At 1 h after the final administration, Neurological deficits were measured according to the method of Bederson [22]. Mice were held suspended above the floor by the tail, and observed for forelimb flexion. Normal mice extend both forelimbs toward the floor. Mice extended both forelimbs toward the floor and that had no other neurological deficit were assigned grade 0. The contralateral forelimb of infarcted mouse was consistently flexed to the injured hemisphere. Mice with any amount of consistent forelimb flexion and no other abnormal behavior were graded 1. Mice were placed on a rough flat surface that could be gripped firmly by their claws. With the tail held by hand, gentle lateral pressure was applied behind the mouse's shoulder. Normal mice resisted sliding equally in both directions. Severely dysfunctional mice had consistently reduced resistance to lateral push toward the paretic side, and were graded 2. Mice were then allowed to move about freely and were observed for circling behavior. Mice that circled toward the paretic side consistently were graded 3. Mice that unable to walk spontaneously and flaccid paralysis were assigned grade 4.

2.6. Measurement of infarct size

Mice were sacrificed and obtain the whole brain sample. Mice brain slicer matrix (Beijing Cinontech Co. Ltd.) was used to obtain consecutive 2 mm thick coronal brain slices. Brain slices were incubated in 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC, Sangon Biotech Co., Ltd.) at 37 °C for 15 min [23]. The unstained area of the brain slice was the infarcted area. The photographed images were analyzed using the Image J software (version, 1.50; National Institutes of Health, Bethesda, Maryland, USA). The five slices were integrated across to determine