Original Article
The protective role of aspirin in patients with acute ischemic stroke

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Abstract: Acute ischemic stroke is regarded as the second leading cause of death. As a primary antiplatelet agent, aspirin has been widely used for the prevention of ischemic stroke. In this study, we aimed to explore the mechanism of aspirin to improve ischemic stroke. The rats were divided into four groups, sham group, MCAO group, MCAO+saline group and Aspirin+MCAO group. After aspirin treatment, the neurological deficits, cerebral Infarct Size and Reactive Oxygen Species (ROS) Production were significantly improved in comparison with saline group. Administration of aspirin significantly reduced the protein level of NOD2. Further study demonstrated the downstream signaling such as NF-κB, and JNK activation was obviously suppressed. Meanwhile, enhanced anti-apoptotic protein, Bcl-2, and decreased pro-apoptotic protein, Bax, can be detected. Consistent with the above data, the levels of TNFα, IL-1β and IL-6 were significantly suppressed in the aspirin group compared with saline group. To conclude, through suppressing NOD2-induced inflammatory responses and ROS production, aspirin treatment could alleviate the deterioration of cerebral function after acute ischemic stroke.

Keywords: Aspirin, acute ischemic stroke, NOD2, inflammation, ROS

Introduction

Acute ischemic stroke is regarded as the second leading cause of death and is often considered as the most common cause of permanent disability among adults in the world [1, 2]. Cerebral ischemia is one of the most important reasons that contributes to stroke [3]. It is reported that the balance between the pro-inflammatory and anti-inflammatory responses can be broken [4]. Further studies reveal that inhibition of inflammatory responses could reduce brain injury and enhance neurological outcome [5, 6]. Thus, prevention of inflammatory responses is especially important for the treatment of acute ischemic stroke.

Innate immunity plays a key role in the first line of defense against infection and many inflammatory responses, including cerebral injury [7, 8]. (NOD)-like receptors (NLRs) are key members that induce inflammatory responses in innate immunity defense [9]. Recent studies indicated the formation of inflamasome complex after focal cerebral ischemia and inhibition of NLRP1 decreased the detrimental injury of post ischemic inflammation [10, 11]. Moreover, deficiency of NLRP3 could improve neurovascular damage after ischemic stroke [12]. Furthermore, the two members of NLR family, NOD1 and NOD2, activates NF-κB signaling through the caspase recruitment domain (CARD) thereby triggering the inflammatory responses [13]. NOD2 was found to be upregulated in microglia and astrocytes after bacteria infectious [14]. However, the specific role of NOD1 and NOD2 has not fully explored after cerebral ischemic injury (CIR).

Aspirin was first discovered by Felix Hoffmann and it can be rapidly absorbed by the stomach and the upper small intestine [15]. As a primary antiplatelet agent, aspirin has been widely used for the prevention of ischemic stroke [16]. Aspirin therapy was estimated to reduce the risk of ischemic stroke by about 15% [17]. However, the specific mechanism in which aspirin improves ischemic stroke has poorly studied. In
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this study, we first determined that aspirin therapy could decrease the expression of NOD2 and reduce NF-κB signaling activation, thereby improving cerebral ischemic injury.

**Materials and methods**

**Animals**

Adult male Sprague-Dawley rats weighing 250-270 g were purchased from the Animal Breeding Center of Sfpanimals Company (Beijing, China). All animals were fed a standard laboratory diet or high fat diet for 10 weeks in a temperature-controlled (20-24°C) and humidity-controlled (45-55%) environment. A 12 h/12 h light/dark cycle was maintained. The animals had free access to food and water. All the experimental procedures were approved by the Animal Care Center of MuDanJiang Medical University. All animal experiments were performed in accordance with the institutional guidelines and ethics.

**Animal models and experimental protocol**

After 24 h of acclimatization, the rats were anesthetized with chloral hydrate at a dose of 400 mg·kg⁻¹ (i.p.). The rectal temperature was maintained at 37 ± 0.5°C throughout the surgical procedure. The MCAO operation by the intraluminal filament method was performed as previously described with some modifications [18]. In brief, 4-0 monofilament nylon suture with a round tip was inserted from the left external carotid artery into the lumen of the internal carotid artery to occlude the origin of the MCA. Aspirin was fed by gavage to rats in experimental group at doses of 2 mg/kg/day for 4 weeks. For control group, water was given only. The rats were randomly divided into the following 4 groups (n = 10/group): sham group; MCAO group; MCAO+saline group; Aspirin+MCAO group. The sham and vehicle-treated rats were injected with physiological saline. Neurological defects were determined at 24 h after the MCAO followed by an examination of the brain infarct volume. The entire brain or the cortex was then removed and processed to detect cerebral infarct size.

**Assessment of neurological defects**

The neurological defects were detected by a single researcher after the MCAO. The researcher was blinded to the experimental treatment groups. The neurological behaviors were scored on 5-point scale as described previously [19].

**Cerebral infarct size**

The cerebral infarct volumes were determined using TTC staining. After treatment, the brains were quickly removed and sliced into 6 coronal sections 40 μm thick. The brain slices were treated with 2% TTC saline solution and incubated at 37.5°C for 30 min, followed by 10% formalin fixation overnight according to a previously described method [20].

**Measurement of reactive oxygen species (ROS) production**

Reactive oxygen species (ROS) production in rat brain mitochondria was monitored by the fluorescent probe DCFH-DA promptly after mitochondria were prepared. Intracellular ROS can oxidize DCFH-DA to dichlorofluorescein (DCF), an intensely fluorescent chemical. Mitochondria isolated from different groups (0.5 mg protein) were incubated with 10 μM DCFAM at 37°C for 60 min, and the fluorescence intensity of DCF was measured at an excitation wavelength of 488 nm and emission wavelength of 527 nm in the microplate reader.

**Western blot**

The rat brain homogenate in ice-cold RIPA buffer (Solarbio, Beijing, China) supplemented with 1 mM PMSF, 1 mM Protease inhibitors and phosphatase inhibitors (Sigma, St. Louis, MO, USA). The homogenate was incubated on ice for 30 min and then centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected, and the protein concentrations of the extracts were measured by BCA assay. The protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes. The membranes were blocked with 8% non-fat milk (EMD Millipore, Billerica, MA, USA) and then washed with PBS for three times (5 min/time). The membrane was then incubated with the following primary antibodies against Bcl-2 (1:1,000), Bax (1:1,000), activated caspase-3 (1:1,000), or PARP (1:1,000) overnight at 4°C. The antibody for β-actin (1:5,000) was used as the internal control. After washing with PBST for three times, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Zhongshanjinqiao, Beijing, China).
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The protein bands were visualized using the ECL Western blotting detection kit (Life Technologies). The relative intensities of the bands were quantified by densitometric analysis.

**Reverse transcription-PCR**

RNA was extracted with RNAvizol reagent (Vigorous, Beijing, China) and reverse transcribed with the EasyScript One-Step gDNA

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**Figure 1.** Aspirin improves neurological defects. A. After 24 h of MCAO, the rats of MCAO group (3.45 ± 0.74) demonstrated higher neurological defects compared with sham group. B. After aspirin treatment, the neurological deficits were significantly improved (1.56 ± 0.34) in comparison with saline group. Data were presented as mean ± SE from 3 independent experiments. *P < 0.05, **P < 0.01.

**Figure 2.** Effects of aspirin on the infarct volume of rats induced by MCAO. A. Representative coronal sections showed that the cerebral infarct size was significantly increased in the serial coronal brain sections of the MCAO rats and administration of aspirin significantly decreased the infarct volume compared with the saline group. B. Statistical analysis of infarct volume. Data were presented as mean ± SE from 3 independent experiments. *P < 0.05, **P < 0.01.
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Removal and cDNA Synthesis SuperMix (AE311-03, Transgene, Beijing, China). The PCR pro-
gram was described as follows: 48°C for 45 min; 96°C for 2 min; 25 repeats of 94°C for 30 s, 55°C for 45 s, and 68°C for 80 s; followed by a final extension at 65°C for 6 min. All PCRs
were run in triplicate and gene expression, relative to GAPDH, was calculated by the compara-
tive ΔΔCt method.

Statistical analyses

The data are represented as means ± standard error (SE) of the indicated number of measure-
ments. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA). P value < 0.05 was considered statistically significant.

Results

Aspirin improves neurological defects

After 24 h of MCAO, the rats of MCAO group (3.45 ± 0.74) demonstrated higher neurological
defects compared with sham group (Figure 1A). After aspirin treatment, the neurological
deficits were significantly improved in comparison with saline group (P < 0.05).

Aspirin improves cerebral infarct size

As shown in Figure 2A, the cerebral infarct size was significantly increased in the serial coronal
brain sections of the MCAO rats. Administration of aspirin significantly decreased the infarct
volume compared with the saline group (Figure 2A and 2B).

Aspirin suppresses the mitochondrial ROS production

Mitochondria are the major sources of ROS production in cells. As shown in Figure 3, ROS pro-
duction was significantly increased in the rats of MCAO group. In comparison, treatment with
aspirin significantly improved ROS production in comparison with the saline group (Figure 3).

Aspirin resulted in the deficiency of NOD2 after cerebral IR injury

It is reported that TLR signaling plays a key in inflammatory responses [21]. NOD2 was
recently found to activate ROS production after cerebral I/R injury [22]. We found the expres-
sion of NOD2 was significantly increased in the rats of MCAO group compared with sham group
(Figure 4A). In comparison, administration of aspirin significantly reduced the protein level of
NOD2 (Figure 4B). Further study demonstrated the downstream signaling, NF-κB, and JNK activa-
tion, was obviously suppressed (Figure 4B). Meanwhile, enhanced anti-apoptotic protein,
Bcl-2, and decreased pro-apoptotic protein, Bax, can be detected (Figure 4B). These data
suggested that aspirin resulted in the deficiency of NOD2 after cerebral IR injury.

Aspirin decreased the I/R induced inflammation

The NF-κB and JNK signaling are key contribu-
tors to the inflammatory responses. Then, we
found that the levels of TNFα, IL-1β and IL-6
were obviously increased in the MCAO group
compared with sham group (Figure 5A). Since
decreased NF-κB and JNK activation was iden-
tified in the aspirin treatment group, we deter-
mined the level of inflammatory factors. The
data suggested that the levels of TNFα, IL-1β
and IL-6 were significantly suppressed in the
aspirin group compared with saline group
(Figure 5B), suggesting the anti-inflammatory
role of aspirin after I/R injury.

Discussion

In this study, we determined that aspirin exerts a protective role on the acute ischemic stroke.
After cerebral ischemic, obvious brain injury,
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Figure 4. Aspirin resulted in the deficiency of NOD2 after cerebral IR injury. A. The expression of NOD2 was significantly increased in the rats of MCAO group compared with sham group. B. Administration of aspirin significantly reduced the protein level of NOD2 and the downstream signaling, NF-κB, and JNK activation. Data were presented as mean ± SE from 3 independent experiments. *P < 0.05, **P < 0.01.

Figure 5. Aspirin decreased the I/R induced inflammation. The levels of TNFα, IL-1β and IL-6 (A) were obviously increased in the MCAO group compared with sham group. The levels of TNFα, IL-1β and IL-6 (B) were significantly suppressed in the aspirin group compared with saline group. Data were presented as mean ± SE from 3 independent experiments. *P < 0.05, **P < 0.01.
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including neurology defects and enhanced cerebral infarct, can be detected. Our results indicated that aspirin could significantly improve brain injury after CIR in the MCAO rat models. Here, we demonstrated that aspirin treatment could obviously decrease NF-κB and JNK activation, thereby suppressing inflammatory responses and cell apoptosis. More importantly, we found that aspirin could decrease the expression of NOD2, thereby alleviating inflammatory damage and ROS production after CIR.

After cerebral ischemic injury, an acute inflammation phase appears with production of large amounts of pro-inflammatory factors [23]. Growing evidences have indicated the important role of NOD2 in the inflammatory signaling after cerebral ischemic injury [24, 25]. In this study, we showed the enhanced expression of NOD2 after CIR. However, whether aspirin could suppress the expression of NOD2 has not been fully studied. Here, we clearly showed that administration with aspirin could markedly down-regulate the protein level of NOD2 in the ischemic brain.

To elucidate the specific mechanism in which NOD2 regulate ischemic injury, we compared the protein expression in the ischemic rat brains compared with the sham group. We found that upregulation of NOD2 could obviously enhance the activation of NF-κB and JNK signaling. The activation of NF-κB and JNK signaling strongly enhance the transcription of pro-inflammatory factors, such as TNFα, IL-1β and IL-6 [26]. In comparison, administration of aspirin could inhibit the activation of NF-κB and JNK signaling. Consistent with decreased NF-κB and JNK activation, aspirin treatment downregulate the expression of TNFα, IL-1β and IL-6.

NOD-derived ROS production is the major origin of cerebral ischemia [24]. Increasing evidence indicates that enhanced ROS production prompts innate immunity responses and enhances cell apoptosis [27, 28]. It has been shown that NOD2 contributes to ROS-induced cell apoptosis. Therefore, it is necessary to determine the levels of ROS production [28]. Obviously, ROS production was enhanced in rats of MCAO group. More importantly, our results demonstrated that aspirin treatment significantly decreased ROS generation and cell apoptosis. Our results showed that aspirin treatment could suppress NOD2-derived ROS production and inflammation.

In summary, this study for the first time showed that aspirin could decrease NOD2 expression in the rats of acute ischemic stroke. Through suppressing NOD2-induced inflammatory responses and ROS production, aspirin treatment could alleviate the deterioration of cerebral function after acute ischemic stroke.

Disclosure of conflict of interest

None.

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