Original Article
The JAK2/STAT3 pathway mediates geranylgeranylacetone-induced neuroprotection against cerebral infarction in rats

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Abstract: Geranylgeranylacetone (GGA) is known as an antiulcer agent and has been reported to have neuroprotective effects against cerebral infarction induced by middle cerebral artery occlusion (MCAO). This study aims to investigate whether the Janus kinase 2 and signal transducer and activator 3 of transcription (JAK2/STAT3) signaling pathway participates in the neuroprotective effects of GGA. Rats were randomly assigned into control group, sham group, MCAO group, MCAO+GGA group, MCAO+AG490 group and MCAO+AG490+GGA group. Infarction volume and neurological deficit scores were used to investigate the neuroprotective effects of GGA. Western blot analysis was used to determine the amount of p-JAK2 and p-STAT3 proteins. The results showed that compared to MCAO, GGA reduced neurological deficits and cerebral infarction volume. In addition, western blot indicated that GGA downregulated the expression of p-JAK2 and p-STAT3 and the downregulation of protein expressions was enhanced by AG490 administration. Our results suggest that GGA protects against cerebral infarction through the suppression of JAK2/STAT3 signaling pathway.

Keywords: Geranylgeranylacetone, cerebral infarction, neuroprotection, JAK2/STAT3 signaling

Introduction

Cerebral infarction is generally defined as an ischemic stroke due to the blockage of the blood vessels supplying blood to the brain [1]. The rising incidence of cerebral infarction is accompanied with the increasing age of the world's population [2]. Besides, cerebral infarction has a serious effect on human health, which can often cause high levels of mortality [3]. Moreover, cerebral infarction is known to be a multifactorial disease induced by complex interactions between genetic and environmental factors [4]. Furthermore, most of the cerebral infarction patients exhibit a specific clinical manifestation of neurologic defects [5]. However, the efficient therapy used to treat cerebral infarction is limited.

Geranylgeranylacetone (GGA) is a derivative of terpenes [6]. Previous studies have demonstrated that GGA has a therapeutic effect on digestive ulcers and portal hypertensive gastropathy [7, 8]. It can penetrate the blood-brain barrier and is centrally active after oral administration [9]. And it promotes neuroprotective effects in response to traumatic brain injury [10]. Increasing evidences have showed that a single oral dose of GGA is a noninvasive pretreatment method for neuronal protection against ischemic insult [11]. However, the signaling pathways in the rat cerebral infarction after GGA administration remain unclear.

The activation of the receptor-associated Janus kinases (JAKs), such as JAK1, JAK2 and JAK3, consequently activate signal transducer and activator of transcription (STAT) proteins [12]. The STAT proteins are a family of transcription factors consisting of seven members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [13]. The activated STAT proteins undergo dimerization and translocation to the nucleus and then regulate the expression of proinflammatory and proapoptotic genes [14]. It has been demonstrated that JAK2/STAT3 activation contributes to neuronal damage after transient focal cerebral ischemia [15]. However,

JAK2/STAT3 also participates in cardioprotection against ischemia-reperfusion injury [16]. Therefore, some controversy exists on whether JAK2/STAT3 signaling is neurotoxic or neuroprotective in ischemic injury [17].

This study aims to investigate the role of JAK2/STAT3 signaling in the neuroprotective effect of GGA against cerebral infarction in rats. We used a rat model for focal cerebral ischemia after middle cerebral artery occlusion (MCAO) to study the neuroprotective role of GGA. In addition, a JAK2 inhibitor AG490 [18] was used to evaluate the effect of inhibiting JAK2 and STAT3 phosphorylation on MCAO-induced infarction and neurological deficit. This is the first report that explores the role of the JAK2/STAT3 pathway in the neuroprotective mechanism of GGA.

Materials and methods

Animals and experimental protocol

All experimental protocols were approved by the Ethics Committee of The First Affiliated Hospital, Zhengzhou University and the experiment protocols were conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Adult male Sprague-Dawley (SD) rats (body weight 240-260 g) were obtained from the Experimental Animal Center of Zhengzhou University.

In one experiment, 36 rats were randomly assigned to 6 experimental groups with 6 rats in each group, including control, sham-operated (sham), MCAO, MCAO+GGA low dose (200 mg/kg), MCAO+GGA middle dose (400 mg/kg) and MCAO+GGA high dose (800 mg/kg) groups. For this experiment, ischemia was induced 48 h after the oral administrations of GGA.

Since a single oral GGA dose (800 mg/kg) 48 h before ischemia significantly alleviated cerebral infarction volume [11], we chose this dose of GGA in the second experiment. 36 rats were randomly assigned to 6 experimental groups with 6 rats in each group, including control, sham-operated (sham), MCAO, MCAO+GGA (800 mg/kg), MCAO+AG490 (intraperitoneal injection with 1 mg/kg AG490 before vehicle administration) and MCAO+GGA+AG490 (intraperitoneal injection with 1 mg/kg AG490 before GGA administration). For this experi-

ment, ischemia was induced 48 h after the oral administrations of GGA. The control and sham groups were given the same dose of vehicle with 5% gum arabic and 0.008% tocopherol 48 h before MCAO.

Establishment of MCAO model

Focal cerebral ischemia was induced by using the traditional transient MCAO model [19]. In brief, the rats were anesthetized with pentobarbital (30 mg/kg). The left carotid artery was exposed to separate the external carotid artery and the internal carotid artery. The middle cerebral artery (MCA) was occluded by inserting a 4-0 monofilament nylon suture (Beijing Sunbio Biotech, China) into the internal carotid artery. The suture was gently advanced until it closed the origin of the MCA. Two hours later, the filament was gently removed. The sham-operated rats received the same surgical without occlusion.

Neurological deficit score

A neurological deficit score was administered by an investigator blinded to the treatment groups at 24 h after MCAO with the scoring scale described previously [20]. Particularly, six tests including spontaneous activity, forepaw outstretching, symmetry in the movement of four limbs, body proprioception, climbing and response to vibrissae touch were performed. Each test was scored from 0 to 3 and then the scores were added up to a score between 0 (severe impairment) and 18 (no neurological impairment).

Infarction volume measurement

Twenty-four hours after MCAO, the brains were sliced into 5 slices with 2-mm thickness. These slices were reacted with 2% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, USA) at 37°C for 15 min, followed by fixation with 4% paraformaldehyde. Then TTC-stained slices were photographed and measured by an image analysis system (NIH Image; National Institutes of Health, USA). And we calculated the infarction areas to gain the infarction volume (mm^3) per brain.

Western blot analysis

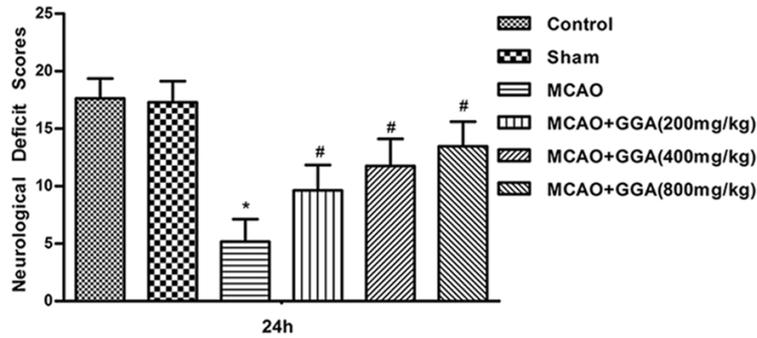For Western blot analysis, the ischemic cortex and corresponding area derived from different

Figure 1. Effects of GGA on neurological deficit scores at 24 h after MCAO. Neurological deficit scores (n = 6 in control, sham and MCAO group, n = 6 in MCAO group pretreatment with 200, 400 and 800 mg/kg GGA). *$P < 0.05$ vs. control group, #$P < 0.05$ vs. MCAO group.
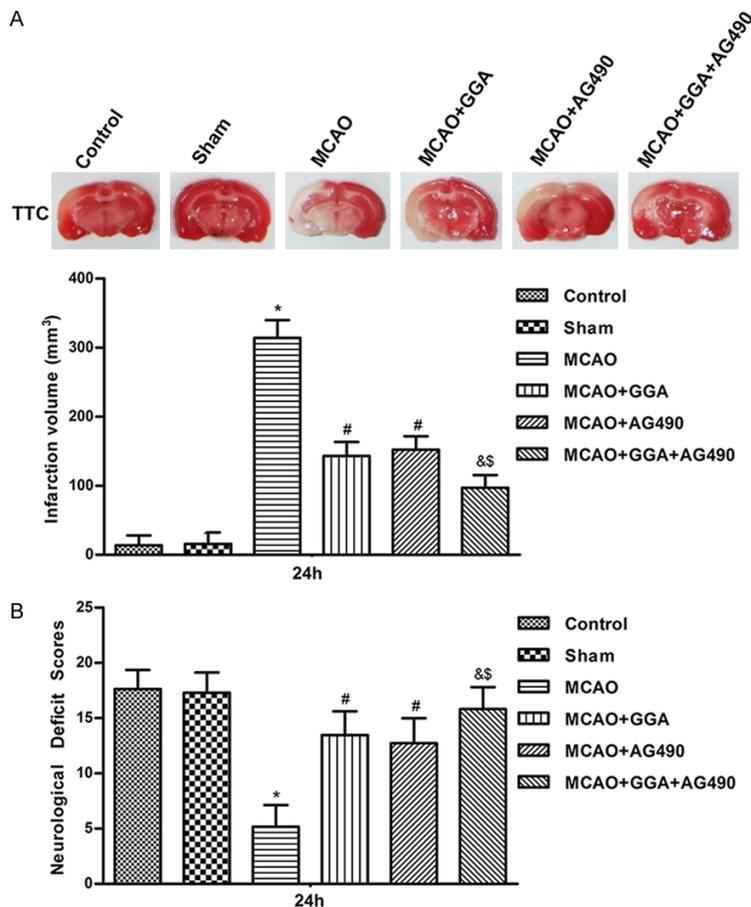

Figure 2. Effects of AG490 treatment combined with GGA on infarction volume and neurological deficit scores after MCAO. A: Photographs of brain slices stained with TTC and quantitative analysis of infarct volume in the six groups. B: Neurological deficit scores in the six groups. *$P < 0.05$ vs. control group, #$P < 0.05$ vs. MCAO group, &$P < 0.05$ vs. MCAO+GGA group, $$P < 0.05$ vs. MCAO+AG490 group.

nized with protein extraction reagent (Pierce, USA) containing a protease inhibitor cocktail (Sigma). Protein concentrations were determined by using a BCA Protein Assay reagent kit (Novagen, USA). Then 50 μg of proteins was separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA) by electrophoresis. After blocked with 0.5% nonfat milk, the membranes were incubated overnight at 4°C with the primary antibodies (anti-JAK2, 1:500; anti-phospho-JAK2, 1:500; anti-STAT3, 1:500; anti-phospho-STAT3, 1:500 and anti-GAPDH, 1:100; Cell Signaling) followed by horse radish peroxidase-conjugated secondary antibody for 1 h at room temperature. Each blot was visualized by an enhanced chemiluminescence detection system (Amersham, UK) and quantified with NIH Image software.

Statistical analysis

All data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL), while all data were expressed as mean ± SD. Multigroup comparisons were carried out using two-way ANOVA followed by Bonferroni *post hoc* tests. Differences with $P < 0.05$ were considered statistically significant.

Results

GGA reduced neurological deficit after MCAO

To determine neuroprotection of GGA against cerebral ischemia insults, MCAO rats were pretreated with different doses of GGA (200, 400 and 800 mg/kg). At 24 h after MCAO, the neurological deficit scores (NDS) decreased significantly

groups were frozen in liquid nitrogen and stored at -80°C. Tissue samples were then homoge-
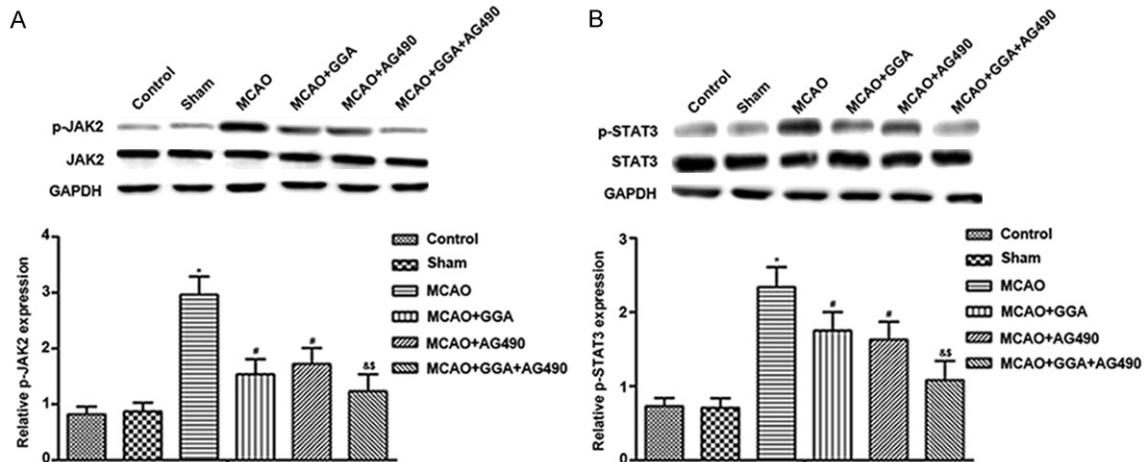
A

B

Figure 3. Effects of AG490 treatment combined with GGA on phosphorylation of JAK2 and STAT3 after MCAO. A: Representative blots and quantitative analyses of p-JAK2 and JAK2 expressions detected by Western blot analysis in the six groups. B: Representative blots and quantitative analyses of p-STAT3 and STAT3 expressions detected by Western blot analysis in the six groups. $^*P < 0.05$ vs. control group, $^\#P < 0.05$ vs. MCAO group, $^\&P < 0.05$ vs. MCAO+GGA group, $^\$P < 0.05$ vs. MCAO+AG490 group.

compared with the control group. However, GGA significantly increased the NDS in a dose-dependent manner after MCAO. In particular, 800 mg/kg GGA pretreated MCAO rats showed a notably reduced neurological deficit (**Figure 1**).

GGA alleviated infarction volume and neurological deficit after MCAO via JAK2/STAT3 pathway

Next, we investigated whether the JAK2/STAT3 signaling pathway was involved in the neuroprotective effects of GGA. As shown in **Figure 2A**, no infarction was observed in control and sham groups. By contrast, obvious lesion was found in both striatum and cortex in MCAO group. In MCAO+GGA or MCAO+AG490 group, infarct volume was significantly reduced compared with that in the MCAO group. And the reduction in infarct volume was enhanced in the MCAO+GGA+AG490 group.

In addition, there was a significant decline in NDS in the MCAO group compared with that in control group. By contrast, compared with the MCAO group, the NDS of GGA or AG490 pretreatment group was much higher. And the increase of NDS was elevated by pretreatment with AG490 before GGA administration (**Figure 2B**).

Effect of GGA on p-JAK2 and p-STAT3 expression

Western blot indicated that JAK2 and STAT3 phosphorylation was dramatically suppressed with GGA or AG490 administration after MCAO. Moreover, the decrease of JAK2 and STAT3 phosphorylation was strengthened by the pretreatment with AG490 before GGA administration (**Figure 3A** and **3B**).

Discussion

GGA, a leading anti-ulcer drug on the Japanese market, has been reported to induce heat shock protein (HSP) expression [21]. Recent studies have shown that GGA could exert protective action against different kinds of stresses in the liver [22], heart [23], glaucoma [24] and gastric mucosa [25]. Currently, increasing reports illustrated the neuroprotective effects of GGA against cerebral infarction. Fujiki et al. reported that GGA reduced neuronal injury induced by transient forebrain ischemia [26]. Nagai et al. evaluated that a single oral dose of GGA played a key role in neuroprotection against cerebral ischemia [11]. Uchida et al. revealed that GGA promoted HSP70 expression in the brain to protect against cerebral infarction in rats [27]. As the neurological deficit score is a common neurological scale in animal studies of stroke [28], an increase in neurologi-

cal deficit score tended to correspond with the degree of neuroprotection in this study. Our results found that 800 mg/kg GGA pretreatment significantly reduced neurological deficit and infarction volume in MCAO rats, which was consistent with previous studies [29]. Thus, GGA plays an important role in neuroprotection against cerebral infarction.

The JAK-STAT pathway, which includes a family of JAKs that activate STATs, can be activated by ischemic injury [30]. Previous studies showed that STAT3 phosphorylation is up-regulated after focal cerebral ischemia in rodents [31]. Additionally, p-JAK2 and p-STAT3 protein expression increased dramatically after cerebral ischemia-reperfusion injury in rats [32]. And our results showed the same tendency after MCAO. Moreover, blocking JAK2/STAT3 activation can decrease renal ischemia-reperfusion injury [33]. In the present work, a JAK2 inhibitor AG490, which has been used to block the phosphorylation of STAT3 in many studies [34], was used to study the role of the JAK2/STAT3 pathway in mediating GGA protection against focal ischemia-induced infarction. We found that AG490 pretreatment enhanced the neuroprotective effects that were conferred by GGA. In addition, the expression of p-JAK2 and p-STAT3 was dramatically lower in the MCAO+ GGA group compared with that in the MCAO group. Interestingly, the reduction in the expression of p-JAK2 and p-STAT3 was strengthened by AG490 administration, suggesting that GGA protects brain ischemic injury via the inhibition of JAK2 and STAT3 phosphorylation.

To our knowledge, this is the first report to demonstrate that GGA pretreatment had a significant protective effect on focal ischemia-induced infarction via the JAK2/STAT3 pathway. And this neuroprotective effect was demonstrated by a reduced neurological deficit and a decreased infarction volume. Moreover, the neuroprotective effect of GGA can be enhanced by AG490, which is a specific inhibitor of the JAK2/STAT3 signaling pathway. Therefore, these results suggest that the neuroprotective effect of GGA is at least partially dependent on the suppression of the JAK2/STAT3 pathway.

In summary, the GGA-induced neuroprotection against cerebral infarction occurs through inhibition of the JAK2/STAT3 signaling pathway. More importantly, GGA may be an attractive new therapy to fight against brain ischemic injuries in clinical use.

Disclosure of conflict of interest

None.

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