RESEARCH PAPER

Acta Neurobiol Exp 2022, 82: 389–397
DOI: 10.55782/ane-2022-037

Isorhapontigenin prevents β-amyloid-associated cognitive impairments through activation of the PI3K/AKT/GSK-3β pathway

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Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease that is the most common cause of dementia in the elderly. Aβ1-42 is significantly associated with memory deficits and it can increase the level of acetylcholine, promote the activity of acetylcholinesterase (AChE), and cause cognitive dysfunction. Isorhapontigenin (ISO) is a stilbene derivative that has antioxidant, anti-tumor, and anti-inflammatory effects. However, it is still unclear whether ISO can affect β-amyloid-associated cognitive impairments. In this study, we found that ISO improved cognitive dysfunction induced by Aβ1-42 in rats. It inhibited the Aβ-induced activation of M1 microglia and reduced the release of inflammatory cytokines. It alleviated amyloid beta-induced oxidative stress and led to an overall improvement in AD symptoms. Cellularly, we found that ISO alleviated Aβ-induced inflammation and oxidative stress by activating the PI3K/AKT/GSK-3β pathway and ultimately improved cognitive dysfunction in AD rats.

Key words: Alzheimer's disease, isorhapontigenin, cognitive dysfunction, PI3K/AKT/GSK-3β pathway, oxidative stress

INTRODUCTION

Alzheimer's disease (AD) is a chronic, progressive neurodegenerative disease that is the most common cause of dementia in the elderly. Senile plaques, which are mainly composed of amyloid protein and neurofibrillary tangles, are typical pathological features of AD (Chang et al., 2010). Among these, the β-amyloid protein (1-42) (Aβ1-42) peptide that is produced by γ-secretase through cleavage of amyloid precursor protein (APP) is closely associated with memory deficits and plays a critical role in increasing the risk of Alzheimer's disease (Dematteis et al., 2020). Aβ1-42 aggregation leads to the loss of the immunoprotective effect of the M2 phenotype of microglia and promotes the polarization of M1 microglia, leading to neuroinflammation and neuronal death (Del Prete et al., 2017). In addition, Aβ can increase the level of acetylcholine (ACh) and promote the activity of acetylcholinesterase (AChE). AChE can in turn increase Aβ deposition and fibrillary formation, and ultimately lead to cognitive dysfunction (Imran et al., 2021).

Isorhapontigenin (ISO) is a stilbene derivative isolated from Chinese traditional medicine Radix (Yao et al., 2021). Many studies have shown that ISO has antioxidant, anti-tumor, and anti-inflammatory effects (Wang et al., 2001; Yao et al., 2004; Wang et al., 2014). For example, ISO has been shown to alleviate osteoarthritis by inhibiting interleukin-1β-induced chondrocyte inflammation and cartilage matrix damage in rats (Yao et al., 2021). It can also improve diabetes in mice by regulating the activity and stability of peroxisome proliferator-activated receptor-γ (PPARγ) in adipocytes (Sun and Cui, 2020). Furthermore, ISO inhibits

Received 28 July 2021, accepted 15 May 2022

inflammatory responses in airway epithelial cells by a corticosteroid-independent mechanism and can be used to treat chronic obstructive pulmonary disease (COPD) (Yeo et al., 2017).

Notably, ISO also has neuroprotective effects (Xu et al., 2016). ISO inhibits oxidative stress and inflammation caused by ischemia and reperfusion, inhibits neuronal cell apoptosis, and protects brain tissue from injury (Ma et al., 2019). ISO can also activate the PI3K/AKT signaling pathway, which is critical for inhibiting Aβ-induced cognitive impairment and AChE activity (Sun and Cui, 2020). Increased activity of the AKT pathway may lead to increased levels of inflammatory factors such as nuclear factor kappa-B (NF-κB). It is unclear whether ISO affects the β-amyloid-associated cognitive impairments. In this study, we found that ISO can improve the cognitive dysfunction induced by amyloid beta protein in rats. It alleviated Aβ-induced inflammation and oxidative stress by activating the PI3K/AKT/GSK-3β pathway. This was associated with reduced cognitive dysfunction in AD model rats.

METHODS

AD model and drug administration

Male Sprague–Dawley rats (12 weeks old, 250–280 g) were bought from Beijing Vital River Experimental Animal Technology (Beijing, China), and maintained in proper temperature and humidity on a 12-h dark/light cycle. The procedures in this study were approved by the Biomedical Ethics Committee at the Medical Department of Xi'an Jiaotong University (Approval no. 2019-383). Forty-eight rats were randomly divided into six groups. To establish the AD model, rats were placed on a stereotaxic frame after being anesthetized with isoflurane. Rats were then implanted bilaterally with a microcannulae in the hippocampi (4.3 mm posterior to the bregma; 3.5 mm lateral from midline; 3.3 mm ventral to bregma). Aβ1-42 was incubated in distilled saline at the concentration of 20 mg/mL for 72 h at 37°C. The scrambled Aβ1-42 peptides were the peptides which have the same size with the Aβ1-42 peptide. Aβ1-42 (China Peptides Co., Ltd, China) or control saline were injected with a microsyringe. The Aβ oligomer (20 μg/3 uL) was injected into the brain. After 10 min, the microinjector was slowly pulled out and the incision site was sutured and disinfected. Isorhapontigenin (purity over 99%) was dissolved in ethanol and diluted with saline to reach an ethanol concentration of 5%. Rats in the sham, scrambled Aβ, and Aβ group received the same volume of 5% ethanol in normal saline. ISO was slowly injected intraperitoneally into the rats at a concentration of 25 mg/kg, 50 mg/kg, and 100 mg/kg for a week, respectively. The scrambled Aβ1-42 sequence was: AIAEGDSHVLKEGAYMEIFDVQGHVFGGKIFRVVDLGSHNVA. The rats were divided into the following experimental groups: sham, scrambled Aβ, Aβ, Aβ+25 mg/kg ISO, Aβ+50 mg/kg ISO, and Aβ+100 mg/kg ISO.

Morris water maze (MWM) tests

To measure learning and spatial memory performance in different groups, the MWM tests were performed in a swimming pool with four quadrants in two diagonals. Black odorless ink was added on the platform to prevent the observation of the position of rats. Rat swimming was recorded by a camera which was linked to a computer-based video system. The rats were trained to find the location of the platform for four consecutive days. The time it took the rats to find the platform was recorded. If the rats were unable to find the location within 90 s, they were placed on the platform to be re-oriented with the surroundings. After the final training, the platform was removed and the number of times and the time it took for the rats to reach the platform were recorded.

Novel object recognition (NOR) tests

The test was separated into three different stages: adaptation, familiarization, and testing. On the first day, the rats were placed in an empty box with no objects and left alone for 5 min. On the second day, the rats were placed in the same box with two identical objects at the same distance with the rats and lasts for 5 min. On the third day, one of the objects was taken away and a new object was placed in the same location. The time it took for the rat to touch two objects within 5 min was recorded.

Immunoblot assay

Proteins were extracted using RIPA buffer (Beyotime). Then, samples were collected and subjected to 10% SDS-PAGE and transferred onto PVDF membranes. Next, membranes were incubated with 5% fat-free milk in TBST buffer to block non-specific binding sites. Subsequently, membranes were incubated with primary antibodies targeting Iba1 (1:1000, Abcam, Cambridge, UK), CD86 (1:1000, Abcam), NF-κB (1:1000, Abcam), p-NF-κB (S536, 1:1000, Abcam), p-tau (S404, 1:1000, Abcam), p-PI3K (Y458, 1:1000, Abcam), PI3K (1:1000, Abcam), p-AKT (T308, 1:1000, Abcam), p-GSK-3β (Y216,

1:1000, Abcam), GSK-3β (1:1000, Abcam), AKT (1:1000, Abcam), AChE (1:1000, Abcam), and beta-actin (1:10000, ab8226, Abcam) for 2 h at room temperature. The membranes were then incubated with specific secondary antibodies at room temperature for 1 h. The blots were then visualized with the ECL kit.

Real-time PCR

Tissues from the injury sites were isolated and used for RNA purification with Total RNA Kit (Tiangen, Beijing, China). Upon measuring RNA concentration and purity, extracted RNA was then used for reverse transcription to generate. Real-time PCR was performed with the SYBR-Green Master Mix (Roche, USA) and respective primers. The following primers were used:

IL-1β: F: TGCCACCTTTTGACAGTGATG,
R: AAGGTCCACGGGAAAGACAC.
TNF-α: F: ACCCTCACACTCACAAACCA,
R: ATAGCAAATCGGCTGACGGT.
β-actin: F: GGAGATTACTGCCCTGGCTCCTAGC,
R: GGCCGGACTCATCGTACTCCTGCTT'.

The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

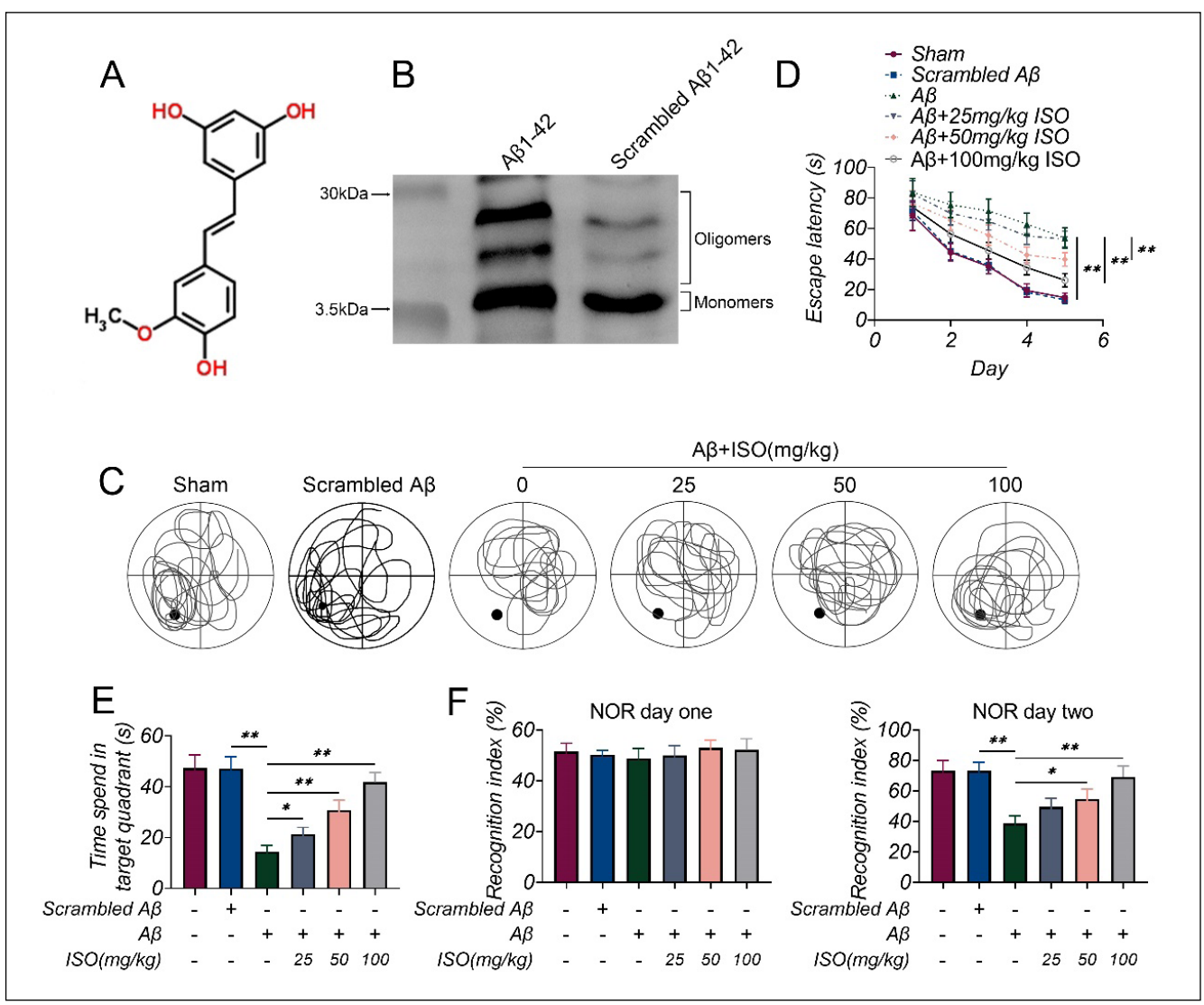

Fig. 1. ISO treatment improves Aβ oligomer-induced cognitive impairments in rats. (A) The molecular formula of ISO. (B, C, D) The effect of ISO on Aβ oligomer-induced cognitive impairments in rats. (D, E) The protective effect of ISO on learning and memory ability. (F) The recognition index (%) of rats upon the indicated treatment in NOR on days one (left) and two (right, n=8) is shown. *P<0.05, **P<0.01.

Assessment of antioxidant activity

The levels of MDA, SOD, GST, and GSH were assessed using detection kits from the Nanjing Jiancheng Bio-engineering Institute (Jiangsu, China) in accordance with the manufacturer's instructions.

IHC staining

Isolated tissues were dehydrated, fixed, and embedded before being cut into 25 μm thick brain slices. Slices were then blocked with goat serum for 1 h. Slices were then incubated with the Aβ antibody (1:200 dilution, Abcam) at 4°C overnight. The next day, samples were washed with PBS and incubated with a fluorescent secondary antibody at room temperature for 1 h. Finally, slices were sealed with anti-quenching agents before observation with a confocal microscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Data was displayed as mean ± SD. Significance was assessed by one-way analysis of variance (ANOVA) and *post hoc* test of comparisons across multiple groups were performed using the Tukey test. $P<0.05$ was considered as statistically significant.

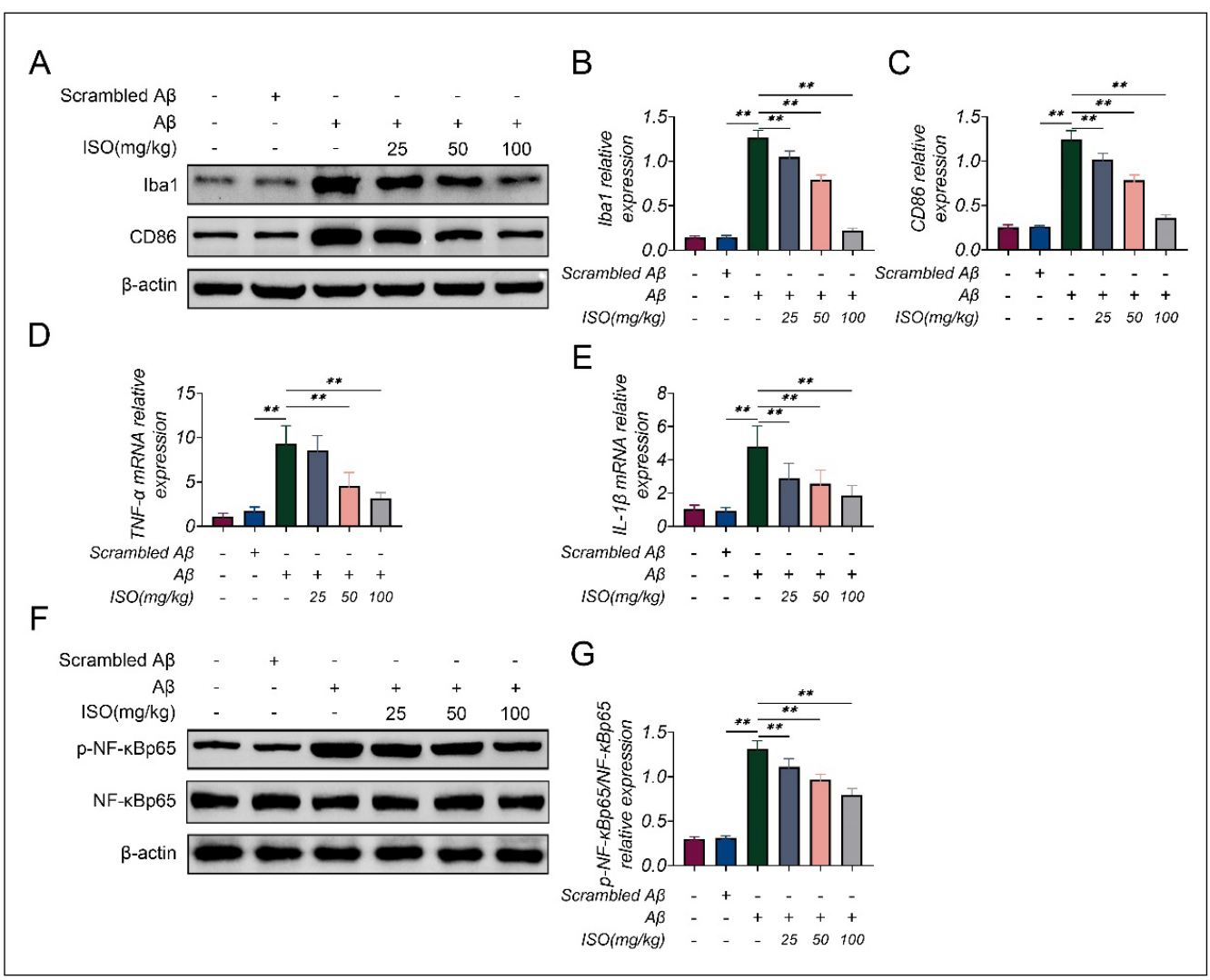

Fig. 2. The treatment of ISO alleviates Aβ-induced inflammation response in the brain. (A, B, C) The expression levels of Iba1 and CD86 in different groups. (D, E) The levels of IL-1β and TNF-α in different groups. (F, G) The levels of p-NF-κB and NF-κB in different groups (n=6). $^{**}P<0.01$.

RESULTS

ISO improves Aβ oligomer-induced cognitive impairments in rats

To detect the effect of ISO on Aβ-induced cognitive impairment in rats, we performed the Morris water maze (MWM) tests in different groups. The molecular formula of ISO is displayed in Fig. 1A. As expected, the cognitive ability in the Aβ group was significantly impaired and rats showed limited ability to find the target as reflected by a longer latency and longer time in the target quadrant. ISO treatment significantly improved the performance of rats in MWM test in a dose-dependent manner ($p<0.05$) (Fig. 1B, C, D). Learning and memory abilities were also measured by the NOR assay. On the first day of the NOR test, the time spent on the exploration of same objects was recorded and the total time was the same across the different groups (Fig. 1E). On the second day, however, the recognition index in the Aβ oligomer treated group was markedly lower than that of the control group. Upon ISO treatment, the recognition index was significantly improved in this group ($p<0.05$) (Fig. 1F). These results indicate that impaired cognitive ability in AD rats can be improved by ISO treatment.

ISO alleviates Aβ-induced inflammation response

Upon evaluating the status of inflammation in the different groups, we noted a major elevation of Iba1 and CD86 levels in Aβ-induced cognitively impaired rats. After ISO treatment, the protein levels were significantly reduced ($p<0.05$) (Fig. 2A, B, C). The levels of IL-1β and TNF-α were also examined by qPCR. The levels of IL-1β

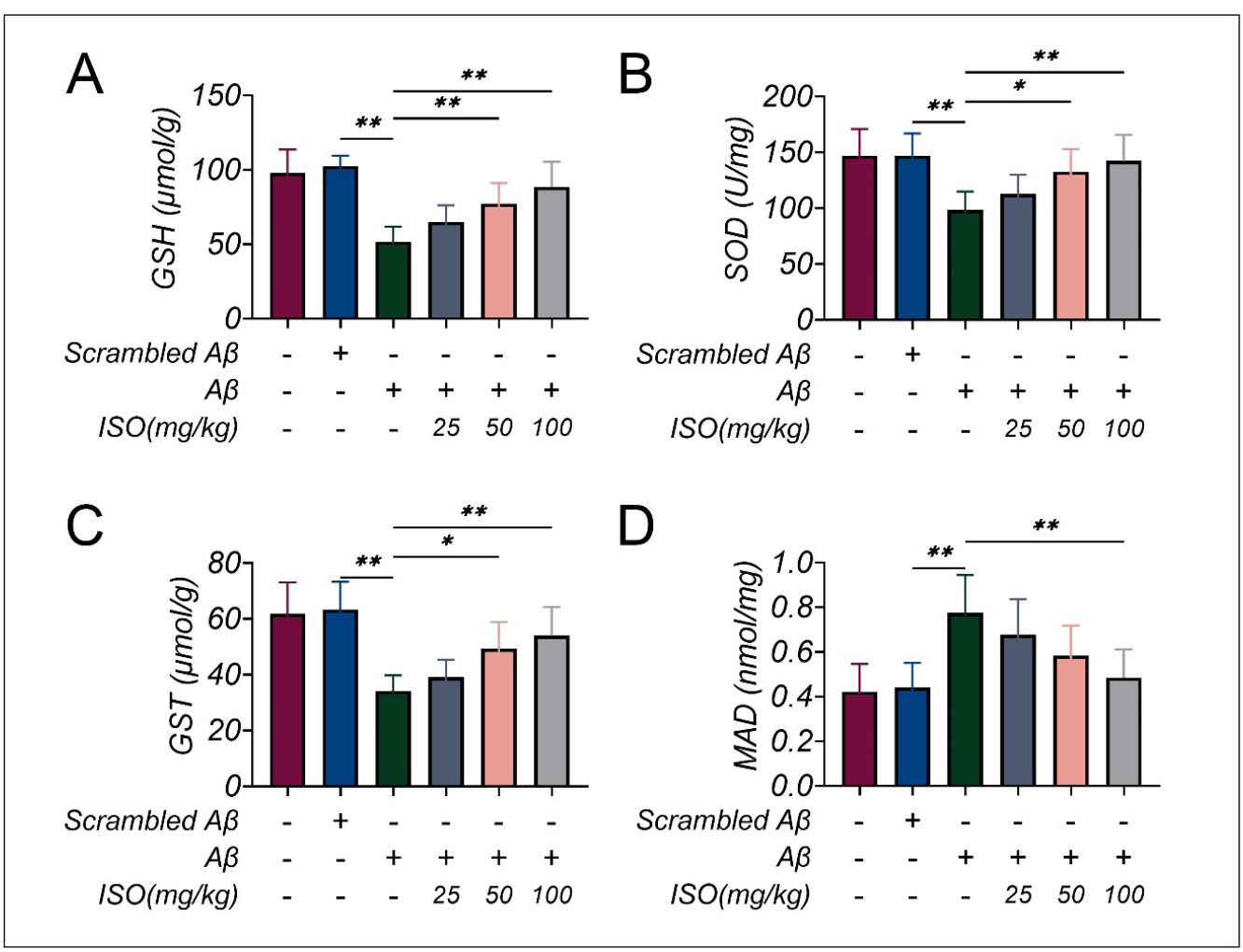

Fig. 3. ISO alleviates Aβ-induced oxidative stress in the brain. (A, B, C, D) The levels of GSH, MDA, SOD and GST in different groups (n=6). *$P<0.05$, **$P<0.01$.

and TNF-α in the Aβ oligomer treated group were higher than those seen in the control group (Fig. 2D, E) and they were subsequently markedly reduced in ISO-treated rats. In addition, in the Aβ oligomer group, there was also an increased expression of p-NF-κB. ISO treatment decreased the level of p-NF-κB, suggesting the inhibition of the inflammatory response (Fig. 2F, G).

ISO alleviates Aβ-induced oxidative stress

Oxidative stress in Aβ-induced rats was detected by measuring the concentration of GSH, SOD, GST, and MDA. The level of MDA was elevated and the levels of GSH, SOD and GST were reduced in Aβ-induced rats ($p<0.05$). ISO treatment significantly decreased the level of MDA and increased the levels of GSH, SOD, and GST ($p<0.05$) (Fig. 3A, B, C, D). These results imply that ISO could exhibit anti-oxidation effects by enhancing levels of endogenous antioxidants.

ISO improves AD symptoms

To measure the effect of ISO on AD symptoms, we evaluated the level of Aβ deposition in the brain. As expected, the deposition of Aβ was significantly increased in the AD group. ISO treatment significantly relieved Aβ deposition ($p<0.05$) (Fig. 4A). The levels of p-tau and AChE were also upregulated in Aβ-induced rats. ISO treatment markedly alleviated the accumulation of p-tau and AChE ($p<0.05$) (Fig. 4B), suggesting some rescue in AD pathology.

ISO alleviates Aβ-induced inflammation and oxidative stress by activating PI3K/AKT/GSK-3β pathway

To uncover of the potential mechanism of ISO-mediated inflammation and oxidative stress, the role of ISO in PI3K/AKT signaling pathway was assessed in each ex-

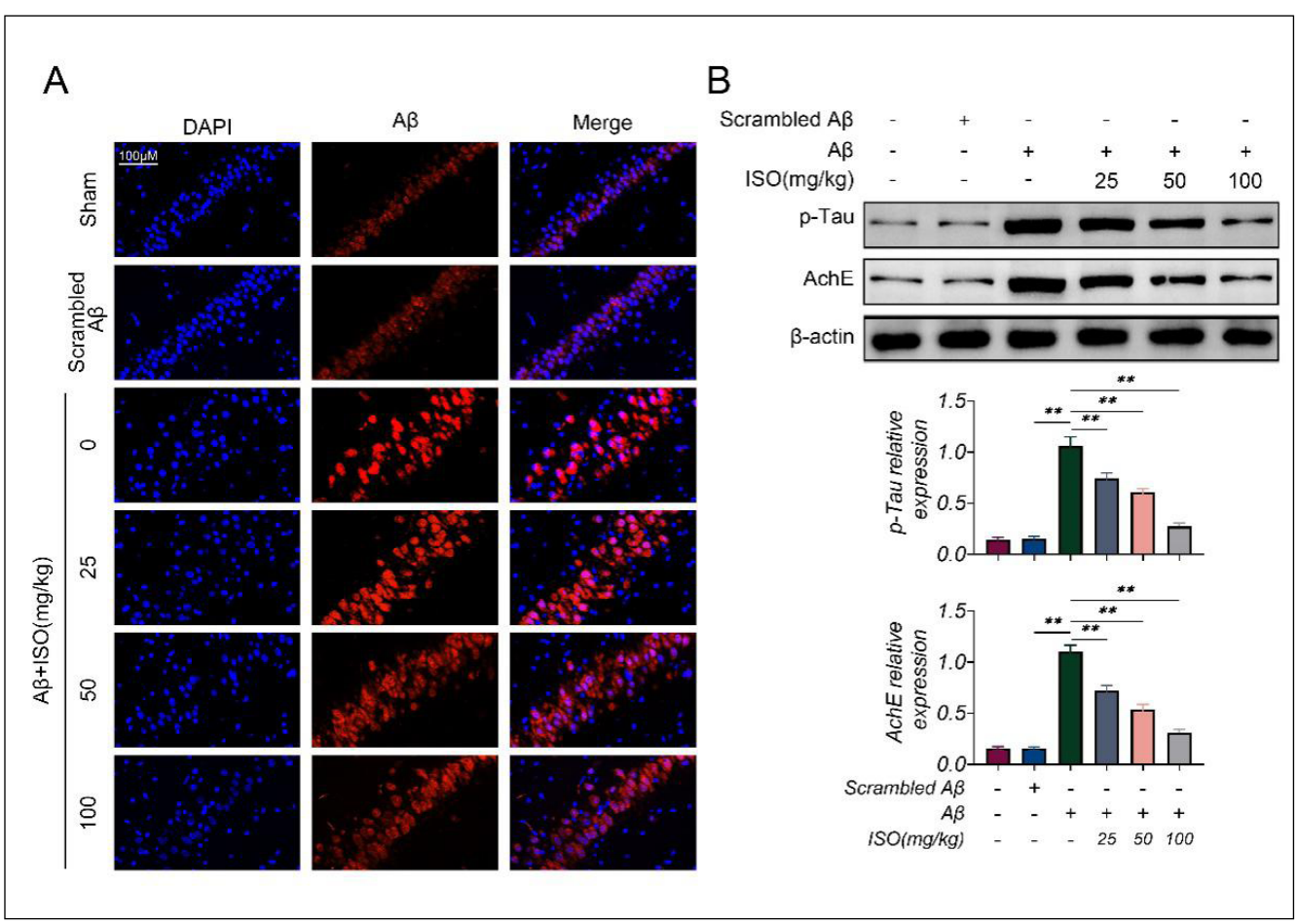

Fig. 4. ISO improves AD symptoms in the brain. (A) The deposition of Aβ in the brain of different groups. (B) The levels of p-tau and AChE in the brains of different groups (n=6). ***P*<0.01.

perimental group. The activation of PI3K/AKT was inhibited in the AD model as demonstrated by decreased levels of phosphorylated PI3K, AKT, and GSK-3β. The repressed levels of phosphorylated PI3K, AKT, and GSK-3β were reversed upon ISO treatment (Fig. 5). This suggests that ISO could alleviate Aβ-induced inflammation and oxidative stress by activating the PI3K/AKT/GSK-3β pathway.

DISCUSSION

AD is a neurodegenerative disease with a rapid onset and progressive worsening of impairments (Gottschalk et al., 2014). Memory impairment and cognitive impairment are the main clinical manifestations. Aβ is closely associated with memory deficits and cognitive impairment, and plays a critical role in increasing the risk of AD dementia (Gurjar et al., 2018). Its aggregation leads to the loss of the immunoprotective effect of the M2 phenotype of microglia, leading to neuroinflammation and neuronal death, and its aggregation also promotes the activity of AChE, which can further contribute to cognitive dysfunction (Jiang et al., 2015). Therefore, it is necessary to improve cognitive function in AD patients. Limiting Aβ-induced inflammation and oxidative stress is one effective strategy that can be used to achieve this (Jouha et al., 2017). Interestingly, here, we found that a promising drug, ISO, showed the potential to treat the cognitive impairment in AD patients. We noticed that ISO could improve Aβ1-42-induced cognitive dysfunction in rats. It alleviated Aβ-induced oxidative stress and improved AD symptoms. We further found that ISO alleviated Aβ-induced inflammation and oxidative stress. Therefore, our data confirms that ISO should be further validated for consideration as a drug for the treatment of cognitive dysfunction in AD patients.

ISO has a variety of biological activities, such as anti-inflammatory and anti-oxidant effects (Sun and Cui,

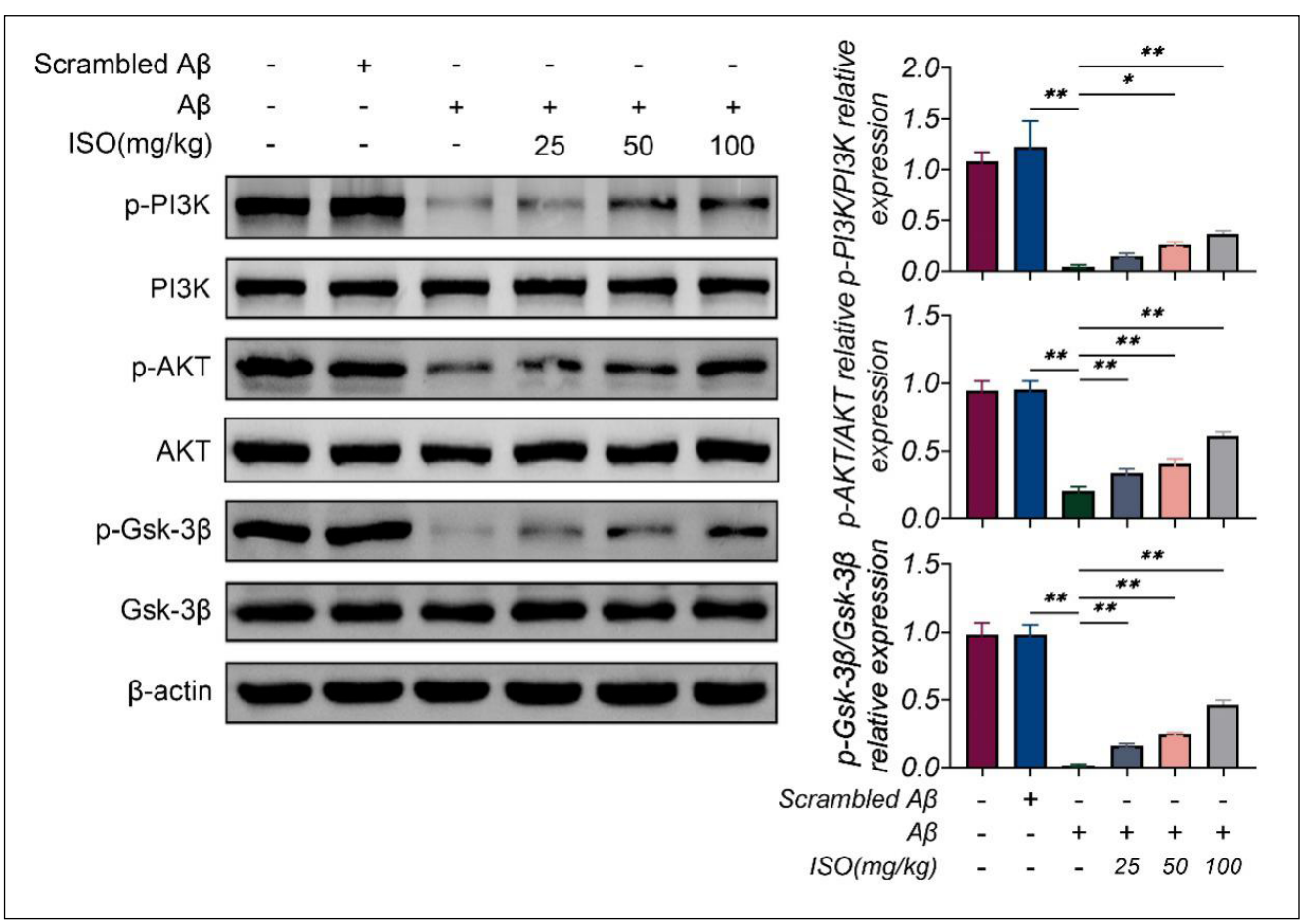

Fig. 5. ISO alleviates Aβ-induced inflammation and oxidative stress by activating the PI3K/AKT/GSK-3β pathway in the brain. The expression levels of phosphorylated PI3K, AKT, GSK-3β and total PI3K, AKT, GSK-3β in the brains of each group. *P<0.05, **P<0.01, ***P<0.001.

2020; Xue et al., 2021). ISO could ameliorate cerebral ischemia/reperfusion injury by mediating the Nrf2/HO-1 pathway (Xue et al., 2021). Isorhapontigenin alleviates lipopolysaccharide-induced acute lung injury by modulating the Nrf2 pathway (Wang et al., 2021). In addition, ISO has been shown to lessen diabetes symptoms by mediating the activity and stability of PPARγ in adipocytes of mice (Chu et al., 2020). ISO also suppresses inflammatory responses in airway epithelial cells through a corticosteroid-independent mechanism and therefore shows potential as a treatment for COPD (Yeo et al., 2017). In this study, we noticed that ISO prevented Aβ-associated cognitive impairments via the PI3K/AKT pathway. Similarly, a previous study indicated that ISO suppresses oxidative stress and inflammation caused by ischemia and reperfusion, inhibits neuronal cell apoptosis, and protects brain tissue from injury. These studies, together with our findings, confirm that ISO could serve as a promising drug for the treatment of neuronal defects and cognitive impairments.

A large number of studies have shown that ISO can activate the PI3K/AKT signaling pathway (Qiu et al., 2020; Wang et al., 2017). Notably, PI3K/AKT activation inhibited Aβ-induced cognitive impairment and inhibited AChE activity (Gu et al., 2020). In addition, AKT can phosphorylate and inhibit the activity of glycogen synthase kinase 3β (GSK-3β) at key Ser9 sites. However, increased GSK-3β activity may lead to enhanced activation of the immune response and increased levels of inflammatory factors such as NF-κB (Qiu et al., 2020). Our data therefore showed that PI3K/AKT/GSK-3β axis could serve as a promising target for the treatment of cognitive dysfunction in AD patients.

CONCLUSION

In conclusion, we found that ISO improved Aβ1-42-induced cognitive dysfunction in rats. It alleviated amyloid beta-induced oxidative stress and alleviated AD symptoms in our animal model. This was found to be related to the activation of the PI3K/AKT/GSK-3β pathway by ISO, which was associated with improvements in cognitive dysfunction in AD rats. Accordingly, ISO could potentially represent a therapeutic option for treatment of cognitive dysfunction in AD patients.

ACKNOWLEDGEMENTS

This work was supported by the Key Research and Development Program of Shaanxi Province (2022SF-527).

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