Diabetes, Metabolic Syndrome and Obesity

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ORIGINAL RESEARCH

Astragaloside IV Improves Cognitive Impairment by Reducing β-Amyloid and Tau Protein Deposition in Hippocampal Tissue of db/db Mice: A PET/CT Imaging-Based Study

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Purpose: Diabetic Cognitive Impairment is a frequent diabetes complication with few treatments. Astragaloside IV (AS-IV), a lanosterol-derived saponin, shows significant neuroprotection. This study used PET/CT to assess the effects of AS-IV on β-amyloid (Aβ) and tau protein buildup in the hippocampus of db/db mice and to investigate the underlying mechanisms involved.

Methods: Eighteen diabetic db/db mice were divided into three groups: a model group, and two treatment groups receiving AS-IV at 20 mg/kg and 40 mg/kg (n=6 each). A control group of db/m mice (n=6) was also included. Treated mice were administered AS-IV daily, while the model and control groups were administered saline for eight weeks. Biweekly, mice were assessed for body weight and fasting blood glucose. Insulin sensitivity was tested using the OGTT, and cognitive function was evaluated with the MWM. Aβ deposition was observed with ^{18}F-AV45 PET and Congo red staining, tau protein deposition with ^{18}F-MK6240 PET, and neurofibrillary tangles with silver staining. TNF-α levels and proteins related to the EGFR/NF-κB pathway were analyzed in blood and hippocampal tissue.

Results: The study found that AS-IV reduced weight gain ($P < 0.05$), lowered fasting glucose ($P < 0.01$–0.001), and improved glucose tolerance ($P < 0.05$–0.001) in db/db mice. Behavioral tests showed that AS-IV treatment decreased escape latency ($P < 0.05$–0.01), increased time in the target quadrant ($P < 0.05$–0.001), and raised the number of platform crossings ($P < 0.05$–0.001). ^{18}F-AV45 PET, ^{18}F-MK6240 PET, Congo red staining and silver staining revealed reduced Aβ ($P < 0.05$–0.001) and tau protein ($P < 0.01$) deposits in the hippocampus. ELISA and immunofluorescence assays indicated a significant decrease in TNF-α expression in serum ($P < 0.05$–0.001) and hippocampus ($P < 0.01$–0.001), while Western blot analyses showed inhibition of the EGFR/NF-κB signaling pathway.

Conclusion: This study found that AS-IV improved cognitive function in diabetic db/db mice by reducing the buildup of Aβ and tau proteins in the hippocampus via the TNF-α-activated EGFR/NF-κB pathway.

Keywords: astragaloside IV, diabetic cognitive impairment, β-amyloid, tau protein, in vivo, PET-CT

Introduction

Diabetes Mellitus (DM) is a metabolic syndrome involving sugars, fats, proteins, water, and electrolytes, resulting from low insulin levels and insulin resistance due to a variety of causes. Epidemiological studies have shown that the global prevalence of DM is rapidly increasing and has become one of the world's major public health problems.[1] The harmfulness and severity of DM are primarily reflected in its various complications. DM can particularly inflict damage on the central nervous system, resulting in alterations in both the function and structure of neural components within the brain. This condition, known as diabetic cognitive impairment (DCI), is characterized by acquired cognitive dysfunction

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and behavioral deficits. Clinically, DCI manifests as a decline in learning and memory, language expression and comprehension, and may be accompanied by apathy and delayed reactions.[2] As a complication of DM, DCI poses significant challenges in terms of prevention and treatment due to its insidious onset and gradual progression, thereby imposing a substantial burden on families and society.[3] Therefore, it is of utmost urgency to uncover the fundamental mechanisms behind DCI and to develop pharmaceuticals capable of alleviating cognitive deficits induced by diabetes.

The pathogenesis of DCI is still unclear. Prior research has demonstrated that this phenomenon is primarily associated with oxidative stress, hyperglycemia, insulin resistance and the accumulation of advanced glycation end products.[4] Epidemiological investigations indicate a concurrent rise in the prevalence of type 2 diabetes and Alzheimer's disease (AD), suggesting a significant correlation between the two conditions.[5] DCI and AD have similar pathological changes, including neurofibrillary tangles (NFTs) resulting from tau hyperphosphorylation and senile plaques (SPs) formed by β-amyloid (Aβ) protein deposits.[6] Excessive accumulation of Aβ can activate microglia, release cytokines, cause neuroinflammation and oxidative stress responses, and lead to neuronal cell dysfunction and even cell death.[7] Imaging studies have demonstrated that Aβ not only elevates tau levels but also facilitates the diffusion of tau, which directly leads to impaired cognitive function and eventually AD.[8] Our previous study validated these results by demonstrating a significant increase in the aggregation of Aβ and tau phosphorylation within the hippocampus of rats suffering from DCI.[9]

Currently, several natural and synthetic compounds have been used in studies related to the treatment of DCI. Resveratrol, a natural polyphenol with antioxidant and anti-inflammatory properties, may improve cognitive function in diabetic patients through multiple signaling pathways.[10] Studies show that berberine exerts its neuroprotective effects through the SIRT1/ER stress pathway, thereby improving diabetic encephalopathy.[11] Additionally, metformin, a commonly used antidiabetic drug, has been found to have potential neuroprotective effects that support cognitive function by improving insulin sensitivity and reducing inflammatory responses.[12] However, the above medications can only alleviate some symptoms and cannot fundamentally curb the development process. Astragaloside IV (AS-IV) serves as the principal active component in total astragaloside. It is found that AS- IV can mitigate oxidative stress, reduce inflammation, prevent apoptosis, enhance immunity and block tumor development, and regulate the activation of the epidermal growth factor receptor (EGFR) signaling pathway.[13,14] Due to its wide range of therapeutic effects, AS- IV has been applied in the treatment of diabetes, cardiovascular disease, kidney disease, and cognitive dysfunction without significant toxic effects.[15,16] In particular, AS- IV can alleviate neuroinflammation and enhance cognitive function in Alzheimer's disease by blocking the nuclear factor kappa-B (NF-κB) signaling pathway.[17] It is inferred from the studies mentioned above that AS-IV may act by regulating the EGFR/NF-κB pathway. Abnormal aggregation and deposition of Aβ and tau proteins are thought to be the primary causes of neuronal damage and cognitive dysfunction. AS-IV has been found to possess antioxidant and anti-apoptotic properties, which may help to attenuate neurotoxicity caused by Aβ and tau.[18] Therefore, we hypothesize that AS-IV ameliorates DCI via downregulation of Aβ and tau accumulation through suppression of TNF-α/EGFR/NF-κB signaling pathway. However, there have been no reports on the effects of AS-IV on the deposition of Aβ and tau proteins in the hippocampus of diabetic db/db mice. Whether AS-IV can inhibit the deposition of Aβ and tau proteins in the hippocampal tissue of mice still needs to be proven. Therefore, this study innovatively used two cognitively related nuclides to evaluate the effects of AS-IV on Aβ and tau deposition in the hippocampal tissues of diabetic db/db mice, and to explore the potential mechanism of action of AS-IV, which will provide insights for the application of AS-IV in the treatment of DCI.

Materials and Methods

Animals

Eight-week-old male diabetic db/db (BKSdb/db, n = 18) and age-matched male nondiabetic db/m mice (BKSdb/m, n=6) were procured from Beijing Viewsolid Biotech Co., Ltd. The mice were kept in a controlled environment at the animal house of Hebei Invivo Biotech Inc, with a circadian rhythm of 12:12 h dark/light, a temperature range between 18 and 25 °C, humidity maintained at 50~60%, and noise levels below 60 dB. Their cages were regularly cleaned and bedding changed. All animal experiments obtained approval from the Ethics Committee of The First Hospital of Hebei Medical University (Approval Number: S00399). The welfare of the laboratory animals was ensured by following the Laboratory

Animal Guidelines for Ethical Review of Animal Welfare (GB/T 35892-2018). All possible measures were taken to reduce pain and distress while limiting the number of animals used in this research.

Groups and Treatments

Eighteen diabetic db/db mice were randomly assigned to three groups: db/db model group, AS-IV 20 mg/kg group, and AS-IV 40 mg/kg group. The normal control group consisted of db/m mice that were raised under identical conditions. AS-IV, with a purity greater than 98%, was obtained from Beijing Solarbio Science & Technology Co., Ltd, located in Beijing, China. Treated mice received daily intragastric administrations of AS-IV (20 or 40 mg/kg), whereas the db/db model and control db/m groups were administered equivalent volumes of saline once daily for eight weeks. Biweekly measurements of body weight and fasting blood glucose levels were performed in experimental mice. The researchers were unaware of the group assignments and drug treatments.

The animals were humanely sacrificed through intraperitoneal injection of sodium pentobarbital (150 mg/kg). Following euthanasia, the brains were promptly isolated and rinsed with saline solution, and then fixed in 4% paraformaldehyde solution in 0.1 M PBS for 3 days. This was done in preparation for the staining procedures involving Congo red, glycine silver, and Immunofluorescence staining. Subsequently, the brains were immersed in paraffin for the purpose of sectioning. The hippocampus was extracted for Western blot and quickly stored at −80°C.

Oral Glucose Tolerance Test (OGTT)

Mice were subjected to an OGTT following eight weeks of AS-IV therapy. Following a 12-hour fast, mice received an intragastric dose of 50% glucose solution at 2.0 g per kilogram of body weight. The glucose concentration in tail blood was assessed at intervals of 0, 30, 60, 90, and 120 minutes with a quick blood glucose meter.[19] The area under the curve (AUC) was determined based on the OGTT results.

MWM Test

The spatial learning and memory functions of the mice were evaluated using the Morris water maze (MWM) test. The MWM apparatus from Shanghai Xinsoft Information Technology Co., Ltd. in Shanghai, China, includes a circular pool, a columnar station with adjustable positioning, a system for tracking animal behavior paths, and a camera setup. During the experimental period, the sink was replenished daily with fresh, white, non-toxic ink water, which was maintained at a temperature of 22–25°C. The MWM positioning navigation test took place during the first five days, with the space exploration test occurring on the sixth day.

Tracer Production and PET Imaging

^{18}F-AV45 and ^{18}F-MK6240 were routinely synthesized at the PET SCIENCE & TECHNOLOGY CO., LTD. (Beijing, China). Mice received isoflurane anesthesia and were intravenously infused with ^{18}F-AV45 (100 ± 20 μCi) or ^{18}F-MK6240 (100 ± 20 μCi). The InliView-3000B, a small-animal PET/SPECT/CT multimodal imaging system from China, was employed to capture static images. The energy window was configured at 80keV. The acquisition involved a 3-minute listmode acquisition followed by 3D rebinning and OSEM reconstruction. The PET images obtained were examined and measured using PMOD software, with data computed within 3D regions of interest (ROIs). Validation of cerebellar reference region: 3D ROIs were sketched for the cerebellum of each group of mice, and the standardized uptake value (SUV) was calculated for 50–60 min static scanning. Analysis showed that the difference in cerebellar SUV between groups was not statistically significant (Figure S1), so the cerebellum was selected as the reference area for hippocampal standardized uptake value ratio (SUVR) calculation.

ELISA Detection

Following behavioral testing, mice were fasted for 12 hours. Serum was collected and stored at −80°C. TNF-α concentrations were quantified using the ELISA kit (VAL630; R&D Systems, USA) according to the manufacturer's protocol.

Congo Red Staining and Glycine Silver Staining

Brain tissue was fixed in 4% paraformaldehyde for 3 days, followed by paraffin embedding and sectioning into 5 μm thick slices. Congo red staining (BA4082A; BASO, China) and glycine silver staining (G1052, Servicebio, China) were conducted following established protocols. The 5 μm paraffin brain sections underwent deparaffinization and rehydration. To perform Congo red staining, sections were immersed in a solution of Congo red for duration of 4 minutes and subsequently washed with tap water for 5 minutes. Afterward, the sections were washed with purified water and then stained with Mayer's hematoxylin solution (BA4082A; BASO, China) for a period of 2 minutes. After another round of rinsing, differentiation was achieved using 0.5% ethanol hydrochloride. Dehydration of the sections was accomplished using anhydrous ethanol, followed by sealing with xylene transparent neutral gum. The bright field images were captured utilizing a Nikon microscope (Eclipse 80i, Japan). In accordance with the manufacturer's protocol, the sections underwent glycine silver staining by being treated sequentially with glycine silver stain C, then B, and finally A. The sections were then dried and covered with a sheet soaked in a blend of ethyl alcohol and xylol, and later examined under a microscope with lighting.

Immunofluorescence Staining

Mouse brain tissue samples were processed for immunofluorescence staining. This method was performed as described previously.[20] The primary antibody used in the present study was TNF-α (1:200, 17590-1-Ig, Proteintech, China). The secondary was goat anti-rabbit IgG (1:200, A24421, Abbkine, USA). The Zeiss LSM 900 confocal microscope (Carl Zeiss, Germany) with either a 10x or 4x objective was used to capture images. Subsequently, Image-Pro Plus software was employed to measure the area of neurons positive for TNF-α. Images were captured using identical settings on the confocal microscope. Three visual fields were randomly picked from the CA1, CA3, and DG areas of the hippocampus for each. Background subtraction was performed using images from unstained areas adjacent to the same section. Following background subtraction, a uniform threshold was applied to all images using Image-Pro Plus software. The percentages of the positive staining were obtained using the area normalization method.

Western Blotting

Total protein was obtained by lysing and collecting the hippocampal tissues from each group of mice. The proteins were quantified using the BCA assay after isolation. A 40 ug protein sample was separated by SDS-PAGE (10% gel) and transferred to a PVDF membrane. Following a 1-hour block with 7% bovine serum albumin (9048-46-8, BioFroxx, Germany) at room temperature, the membranes were incubated overnight at 4°C with specific primary antibodies targeting β-actin (1:2000, 20536-1-AP, Proteintech, China), p-EGFR (1:1000, AF3048, Affinity Biosciences, China), EGFR (1:1000, AF6043, Affinity Biosciences, China), p-NF-κB (1:1000, MA5-15160, invitrogen, USA) and NF-κB (1:1000, AF5006, Affinity Biosciences, China). Subsequently, the membranes were incubated with fluorescein-conjugated goat anti-rabbit IgG DyLight 800 (1:2000, A23920, Abbkine, China) and goat anti-mouse IgG Dylight 680 (1:2000, A23710, Abbkine, China) for 40 minutes at room temperature. The Odyssey® CLx imaging system (LI-COR Biosciences, USA) was utilized for detection, and the Image-Pro plus software was employed to determine the optical density values of the target band. Normalization of the target protein to β-actin ratio was done relative to the negative control. The three samples in each group were collected independently from three different individual mice.

Statistical Analysis

The data were examined utilizing GraphPad Prism v.9.5.0 software for statistical analysis and displayed as the average ± standard error of the mean. The Shapiro–Wilk test was employed to assess normality. If the experimental data exhibited normal distribution and met the assumption of equal variances, one-way ANOVA was employed to compare means across multiple groups. In the case of one-way ANOVA analysis, the Tukey's test was utilized for post hoc multiple comparisons. For data not fitting a normal distribution, M (P 25, P 75) was used for expression, and the Kruskal–Wallis H-test with Dunnett correction compared group differences. Statistical significance was determined at $p < 0.05$.

Results

AS-IV Reduced Weight Gain, Lowered Fasting Glucose Levels, and Improved Glucose Tolerance in db/db Mice

During the 8-week feeding period, db/db mice, which served as a model for spontaneous diabetes, exhibited significantly elevated body weight and fasting blood glucose levels compared to the db/m control mice. These findings align with the typical glucose and body weight profiles observed in type 2 diabetes models. After 8 weeks of oral gavage treatment of db/db mice in the administration groups, there were different degrees of improvement in body weight gain among the treated groups. The group receiving AS-IV at 40 mg/kg exhibited the most notable enhancement relative to the model group (47.87 ± 3.38g versus 54.83 ± 5.60g, n = 6; $p < 0.05$) (Figure 1A). The blood glucose levels of db/m control mice was maintained at a normal range (5.3~6.5 mmol/L), whereas the blood glucose in the model group continuously increased. In comparison to the db/db model group, treatments with AS-IV at doses of 20 mg/kg and 40 mg/kg notably lowered fasting blood glucose levels at weeks 4 (20 mg/kg AS-IV, 19.75 ± 1.46 mmol/L versus 23.57 ± 2.51 mmol/L, n = 6; $p < 0.01$) (40 mg/kg AS-IV, 15.95 ± 1.03 mmol/L versus 23.57 ± 2.51 mmol/L, n=6; $p < 0.001$), 6 (20 mg/kg AS-IV, 17.75 ± 1.47 mmol/L versus 24.73 ± 2.58 mmol/L, n = 6; $p < 0.001$) (40 mg/kg AS-IV, 15.58 ± 1.13 mmol/L versus 24.73 ± 2.58 mmol/L, n = 6; $p < 0.001$), and 8 (20 mg/kg AS-IV, 17.27 ± 1.75 mmol/L versus 27.12 ± 2.43 mmol/L, n = 6; $p < 0.001$) (40 mg/kg AS-IV, 15.02 ± 1.03 mmol/L versus 27.12 ± 2.43 mmol/L, n = 6; $p < 0.001$) (Figure 1B). In the OGTT, the mean blood glucose levels of db/m mice peaked at 30 min and then rapidly declined to normal levels. In contrast, the average blood glucose level of db/db mice peaked at 30 min and then remained elevated, indicating glucose intolerance (Figure 1C). However, after 8 weeks of continuous dosing, treated db/db mice exhibited significantly lower blood glucose levels at 60 (20 mg/kg AS-IV, 27.10 ± 3.36mmol/L versus 30.62 ± 4.07mmol/L, n = 6; $p > 0.05$) (40 mg/kg AS-IV, 24.00 ± 3.72mmol/L versus 30.62 ± 4.07mmol/L, n = 6; $p < 0.05$), 90 (20 mg/kg AS-IV, 23.62 ± 3.18mmol/L versus 28.78 ± 3.53mmol/L, n = 6; $p < 0.05$) (40 mg/kg AS-IV, 19.92 ± 2.16mmol/L versus 28.78 ± 3.53mmol/L, n = 6; $p < 0.001$), and 120 (20 mg/kg AS-IV, 21.72 ± 2.93mmol/L versus 25.58 ± 3.50mmol/L, n = 6; $p > 0.05$) (40 mg/kg AS-

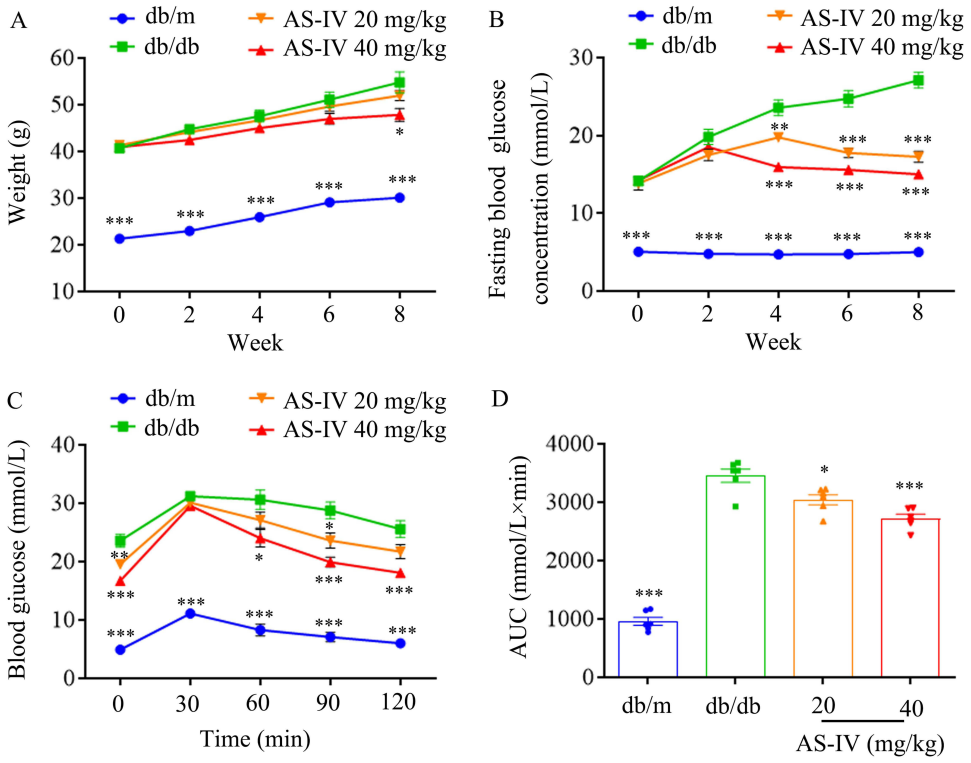

Figure 1 The effects of AS-IV on body weight, fasting blood glucose, and glucose tolerance in db/db mice. (**A**) Body Weight of mice in each group during the 8-week treatment period. (**B**) Fasting blood glucose levels in mice from each group throughout the 8-week treatment. (**C**) Curve of blood glucose level in OGTT test. (**D**) Glucose total AUC in OGTT test. Values represented as means ± SEM (n = 6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the model group.

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IV, 18.08 ± 1.59mmol/L versus 25.58 ± 3.50mmol/L, n = 6; $p < 0.001$) minutes post-glucose administration compared to untreated db/db model mice. This effect was observed at doses of 20 mg/kg and 40 mg/kg, and the area under the glycemic curve was correspondingly reduced (20 mg/kg AS-IV, 3,044.0 ± 210.4mmol/L×min versus 3,455.0 ± 277.2mmol/L×min, n = 6; $p < 0.05$) (40 mg/kg AS-IV, 2,727.0 ± 175.8mmol/L×min versus 3,455.0 ± 277.2mmol/L×min, n = 6; $p < 0.001$) (Figure 1D).

AS-IV Enhanced Spatial Learning and Memory in db/db Mice

To investigate the impact of AS-IV on cognitive function in db/db mice, we assessed spatial learning and memory using the MWM test after 8 weeks of treatment. MWM test results showed that from the fourth day onwards, db/db model mice had a notably longer escape latency compared to db/m control mice (Training day 4, 11.04 ± 6.33s versus 29.43 ± 16.25s, n = 6; $p < 0.05$) (Training day 5, 6.18 ± 1.83s versus 23.81 ± 15.05s, n = 6; $p < 0.01$), but treatment with 40 mg/kg AS-IV significantly decreased this latency (Training day 4, 17.09 ± 3.99s versus 29.43 ± 16.25s, n = 6; $p < 0.05$) (Training day 5, 9.72 ± 2.87s versus 23.81 ± 15.05s, n = 6; $p < 0.05$) (Figure 2A and B). On the 6th day of the spatial probe test, db/db mice showed less time exploring the target quadrant (III) and markedly lower crossing platform times than db/m mice (Time spent in quadrants III, 27.54 ± 4.69s versus 10.23 ± 5.11s, n = 6; $p < 0.001$) (Crossing platform times, 9.67 ± 2.66s versus 3.83 ± 2.14s, n = 6; $p < 0.001$). This indicated that the memory abilities of db/db mice was impaired. Compared to db/db model mice, both AS-IV 20 mg/kg (Time spent in quadrants III, 18.50 ± 2.97s versus 10.23 ± 5.11s, n = 6; $p < 0.05$) (Crossing platform times, 6.67 ± 2.07s versus 3.83 ± 2.14s, n = 6; $p > 0.05$) and 40 mg/kg (Time spent in quadrants III, 22.41 ± 4.42s versus 10.23 ± 5.11s, n = 6; $p < 0.001$) (Crossing platform times, 7.83 ± 1.60s versus 3.83 ± 2.14s, n=6; $p < 0.05$) treatments significantly alleviated the decline in the aforementioned indicators (Figure 2C and D). These findings demonstrate that AS-IV treatment significantly enhanced cognitive performance in db/db mice.

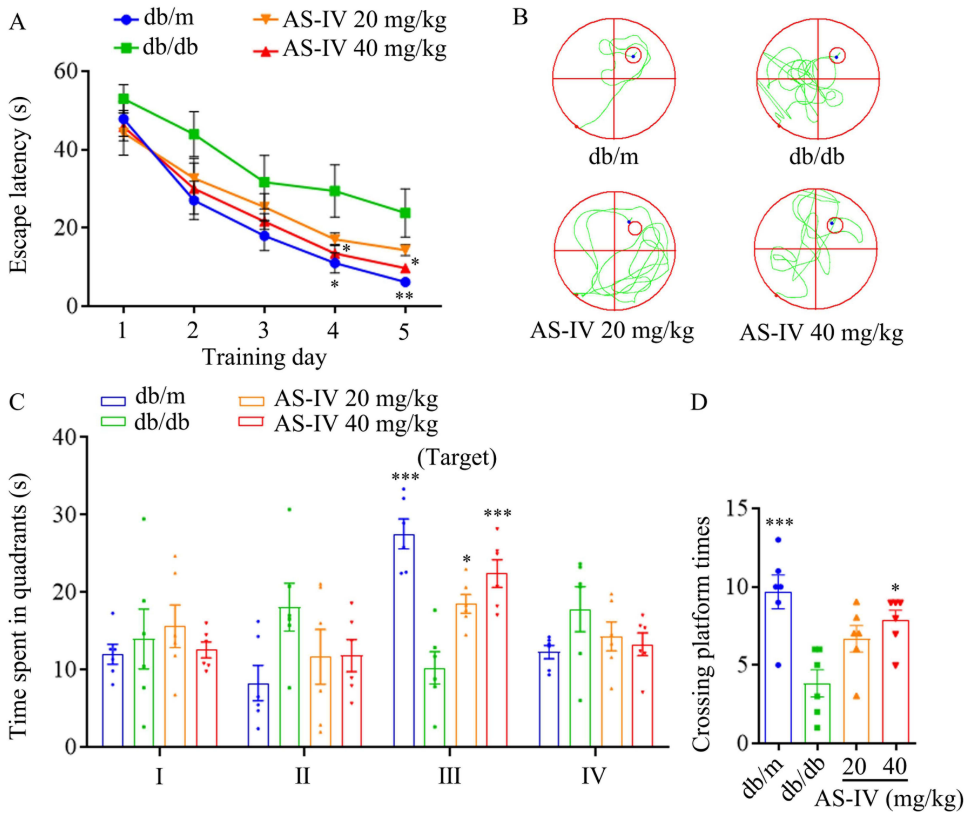

Figure 2 The effects of AS-IV on cognitive function in db/db mice. (**A**) The escape latencies of mice across various groups over five consecutive training days. (**B**) Typical swimming paths for finding the platform. (**C**) Time spent in the four quadrants was measured on day six. (**D**) The crossing platform numbers in different groups on the sixth day. Values represented as means ± SEM (n = 6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the model group.

AS-IV Reduced Aβ Protein Deposition in Hippocampal Tissues of db/db Mice

In order to examine the influence of AS-IV on Aβ protein deposition in hippocampal tissue, we injected ^{18}F-labeled AV45 through the tail vein and performed in vivo imaging using PET. All mice successfully completed the ^{18}F-AV45 PET/CT scans, and no animals were excluded. The imaging findings were validated by the congo red staining. The ^{18}F-AV45 PET/CT results showed no statistically significant differences in cerebellar Aβ protein deposition among the groups of mice (Figure S1A). ^{18}F-AV45 PET/CT imaging (Figure 3A–C) and congo red staining (Figure 3D and E) results revealed that, Aβ protein deposition in the hippocampal tissue of db/db model mice was significantly higher than in db/m control mice (^{18}F-AV45 PET/CT SUVR, 0.45 ± 0.16 versus 1.30 ± 0.08, n = 6; $p < 0.001$) (The numbers of Aβ-positive dots in the hippocampus, 8.00 ± 4.36 versus 56.33 ± 11.37, n = 3; $p < 0.001$), particularly in the DG, CA1, and CA3 regions. Notably, both the 20 mg/kg (^{18}F-AV45 PET/CT SUVR, 1.09 ± 0.13 versus 1.30 ± 0.08, n = 6; $p < 0.05$) (The numbers of Aβ-positive dots in the hippocampus, 33.67 ± 4.51s versus 56.33 ± 11.37, n = 3; $p < 0.05$) and 40 mg/kg (^{18}F-AV45 PET/CT SUVR, 0.91 ± 0.09 versus 1.30 ± 0.08, n = 6; $p < 0.001$) (The numbers of Aβ-positive dots in the hippocampus, 20.33 ± 6.03s versus 56.33 ± 11.37, n = 3; $p < 0.01$) AS-IV treatments significantly decreased the Aβ protein deposition in hippocampal tissue of db/db model mice. These results indicated that AS-IV reduced Aβ protein deposition in hippocampal tissues of db/db mice.

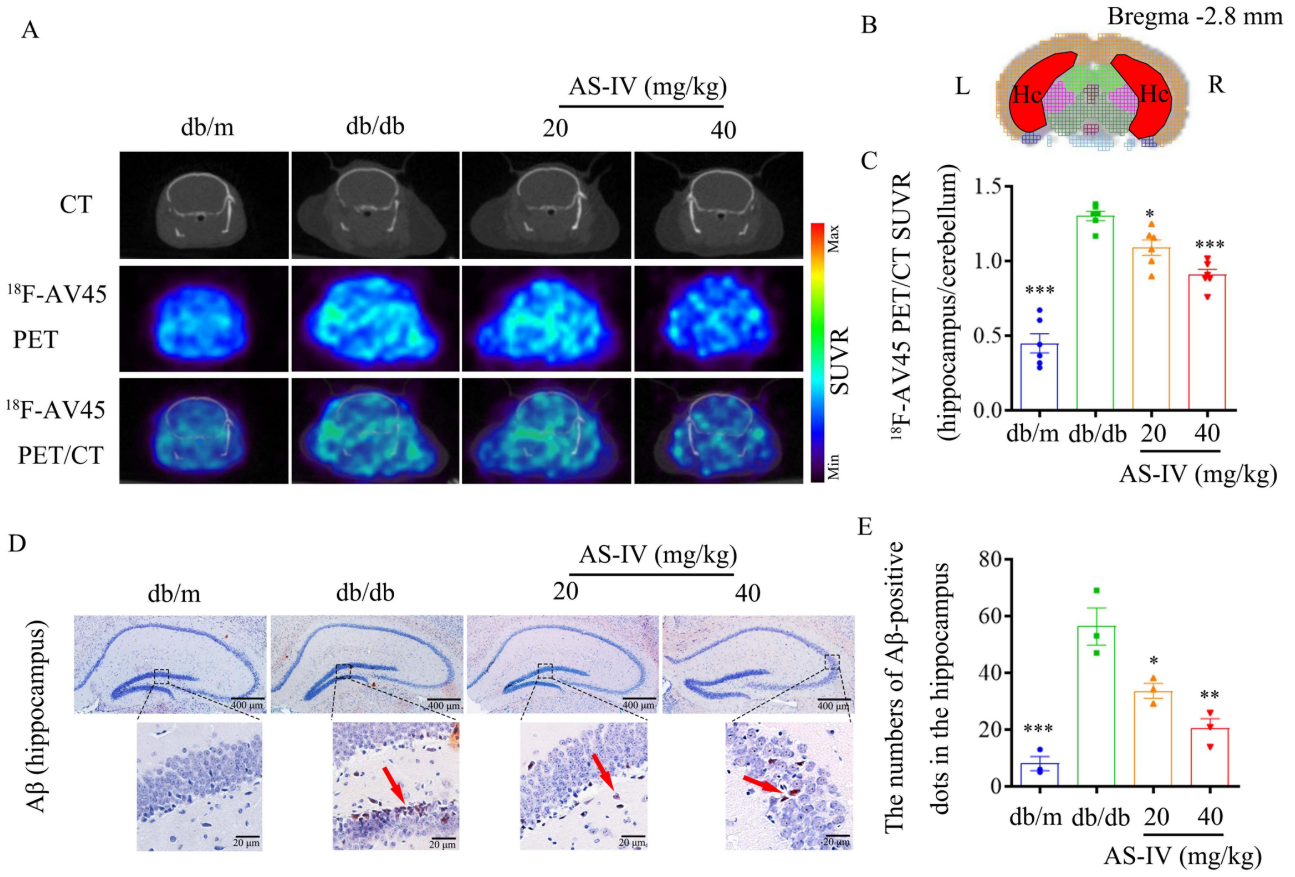

Figure 3 The effects of AS-IV on the expression of Aβ in the hippocampus of db/db mice. (**A**) Representative ^{18}F-AV45 PET/CT images in the hippocampus for each mouse group. (**B**) Representative coronal ^{18}F-AV45 PET/CT images of the mice obtained from bregma –2.8 mm. (**C**) Region of interest (ROI)-to-cerebellum ratios for each group of mice based on ^{18}F-radioactivity data. (**D**) Congo red staining of the hippocampus (red arrow). (**E**) The numbers of Aβ-positive dots in the hippocampus for each mouse group. Values represented as means ± SEM (n = 6 or 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the model group.

Abbreviations: Hc, Hippocampus; L, left; R, right.

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AS-IV Reduced Tau Protein Deposition in Hippocampal Tissues of db/db Mice

We injected ^{18}F-labeled MK6240 through the tail vein and used PET imaging to investigate the effects of AS-IV on tau protein accumulation in hippocampal tissue. All mice successfully completed the ^{18}F-MK6240 PET/CT scans, and no animals were excluded. The imaging findings were validated by the glycine silver staining. The ^{18}F-MK6240 PET/CT results showed no statistically significant differences in cerebellar tau protein deposition among the groups of mice (Figure S1B). ^{18}F- MK6240 PET/CT imaging (Figure 4A and B) results showed that the deposition of tau protein in the hippocampal tissue of db/db model mice was significantly increased compared with db/m control mice (0.71 ± 0.12 versus 1.33 ± 0.48, n = 6; $p < 0.01$). AS-IV 40 mg/kg treatment remarkably reduced the tau protein deposition in hippocampal tissue of db/db model mice (0.73 ± 0.17 versus 1.33 ± 0.48, n = 6; $p < 0.01$). Glycine silver staining results showed an increase in neurofibrillary tangles in the hippocampal tissue of db/db mice compared to db/m mice (Figure 4C). Both AS-IV 20 mg/kg and 40 mg/kg treatments significantly reversed this process. The findings suggested that AS-IV decreased tau protein accumulation in hippocampal tissues of db/db mice.

AS-IV Reduced TNF-α Expression in Blood and Hippocampal Tissue of db/db Mice

As a proinflammatory mediator, TNF-α can induce a systemic inflammatory response.[21] ELISA and immunofluorescence staining were employed to measure TNF-α expression in the serum and hippocampus of db/db mice. According to the ELISA test results, db/db mice had higher serum levels of TNF-α than db/m mice (297.80 ± 45.53ng/L versus 525.70 ± 39.0ng/L, n = 6; $p < 0.001$) (Figure 5A). Administering 20 mg/kg and 40 mg/kg of AS-IV led to a notable reduction in

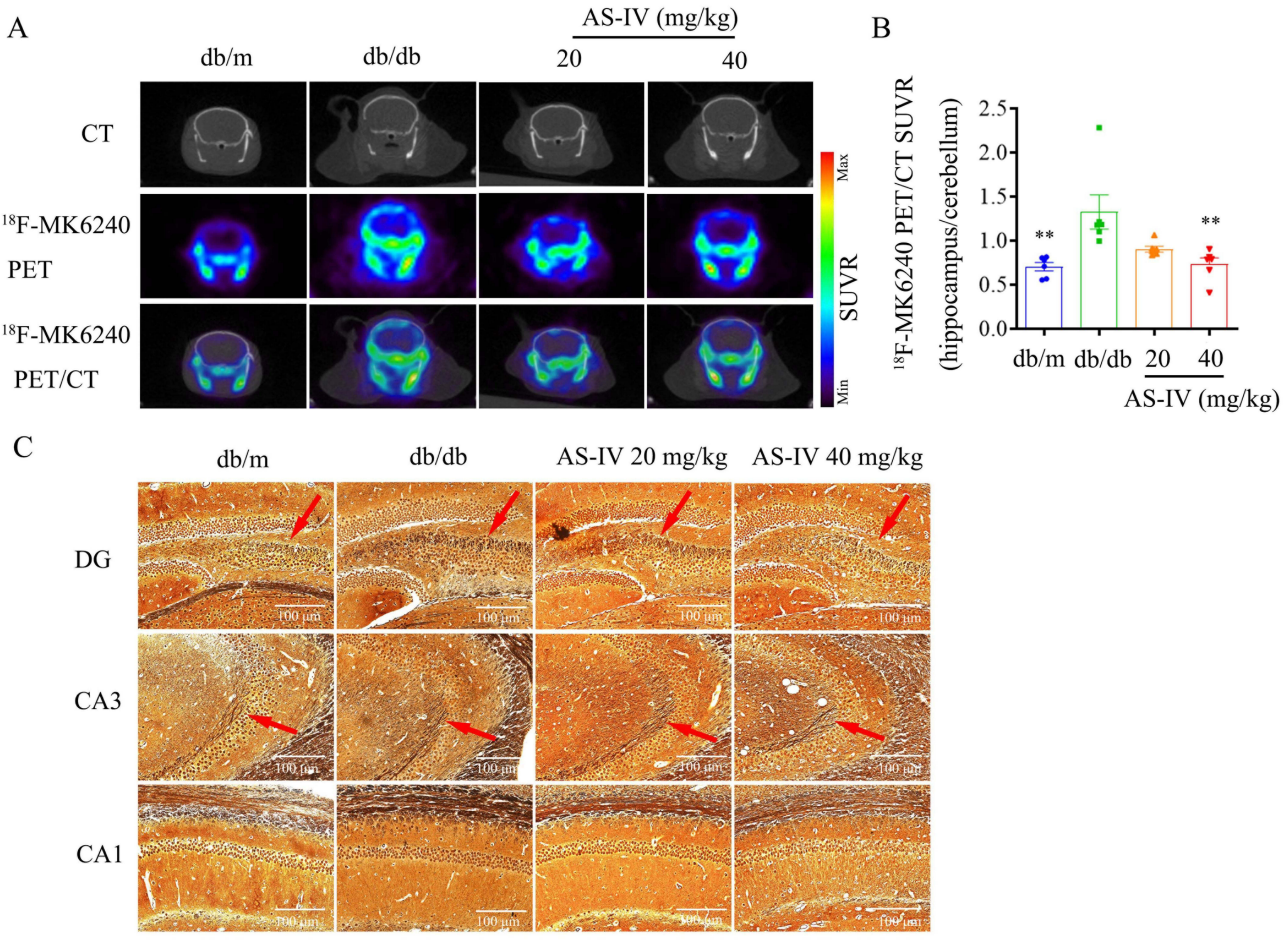

Figure 4 The effects of AS-IV on the expression of tau protein deposition and neurofibrillary tangles in the hippocampus of db/db mice. (**A**) Representative coronal ^{18}F-MK6240 PET/CT images in the hippocampus of each group of mice (bregma –2.8 mm). (**B**) Region of interest (ROI)-to-cerebellum ratios for each group of mice based on ^{18}F-radioactivity data. (**C**) Glycine silver staining of the hippocampus (red arrow). Values represented as means ± SEM (n = 6). $^{**}P < 0.01$, compared with the model group.

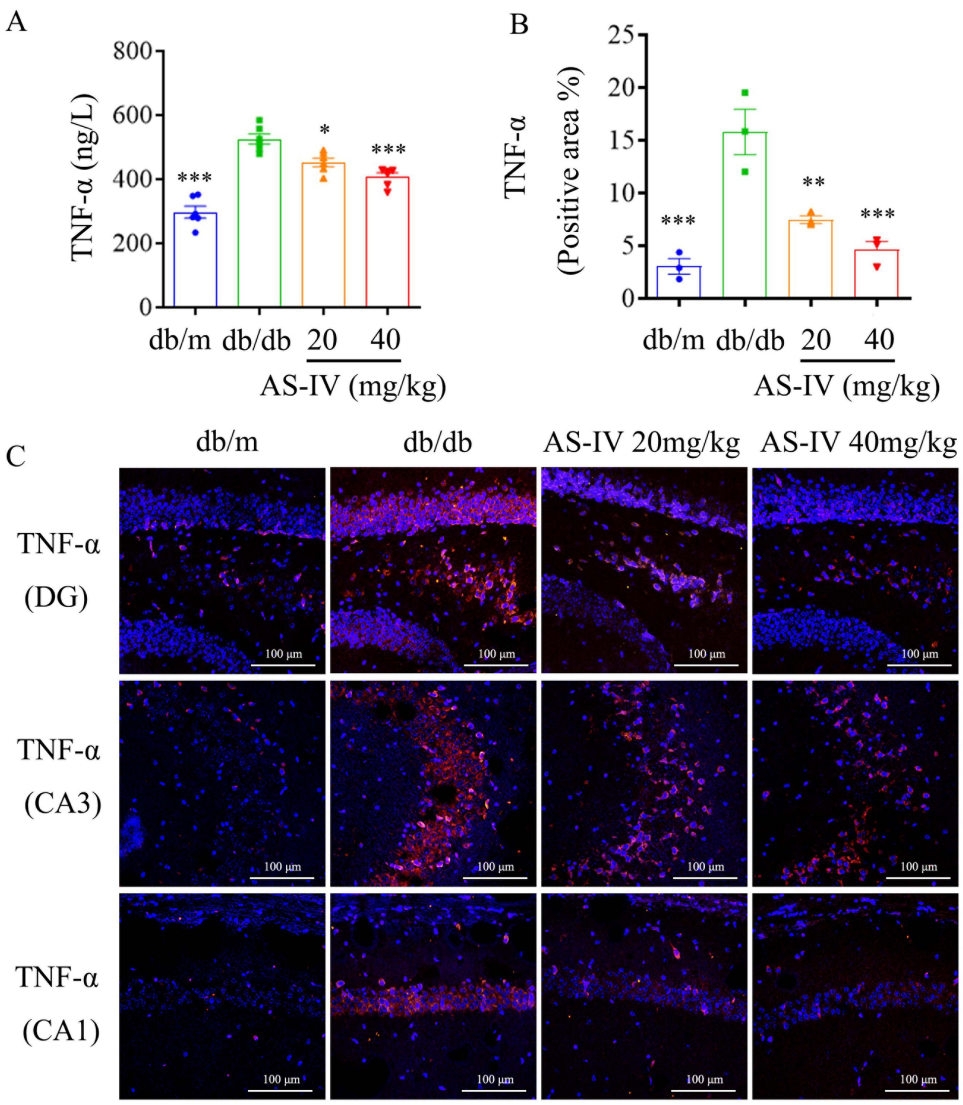

Figure 5 The effects of AS-IV on the expression of TNF-α in the serum and hippocampus of db/db mice. (**A**) The relative expressions of TNF-α in mice blood. (**B**) The quantification for positive area of TNF-α in mice hippocampus. (**C**) Immunofluorescence staining of TNF-α in the hippocampus for each mouse group. Values represented as means ± SEM (n = 6 or 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the model group.

serum TNF-α levels in db/db mice (20 mg/kg AS-IV, 452.50 ± 32.52ng/L versus 525.70 ± 39.0ng/L, n = 6; $p < 0.05$) (40 mg/kg AS-IV, 408.50 ± 28.68ng/L versus 525.70 ± 39.0ng/L, n=6; $p < 0.001$). However, the levels did not completely revert to those seen in db/m mice. Immunofluorescence staining (Figure 5B and C) showed that TNF-α expression was significantly elevated in the hippocampal DG, CA1, and CA3 regions of db/db mice compared with db/m mice (3.04 ± 1.28% versus 15.79 ± 3.75%, n = 3; $p < 0.001$). The process was significantly counteracted by AS-IV treatments of 20 mg/kg (7.47 ± 0.63% versus 15.79 ± 3.75%, n = 3; $p < 0.01$) and 40 mg/kg (4.61±1.37% versus 15.79 ± 3.75%, n = 3; $p < 0.001$).

AS-IV Inhibited the Activation of the EGFR/NF-κB Pathway in Hippocampal Tissues of db/db Mice

To explore the impact of AS-IV on the EGFR/NF-κB pathway in db/db mice, the hippocampal protein expression levels of p-EGFR, EGFR, p-NF-κB, and NF-κB were quantified. Our results show a notable rise in the phosphorylation levels of EGFR (0.99 ± 0.02 versus 2.98 ± 0.46, n = 3; $p < 0.001$) (Figure 6A) and NF-κB (0.99 ± 0.02 versus 2.54 ± 0.58, n = 3; $p < 0.01$) (Figure 6B) in the hippocampal tissues of db/db model mice when compared to db/m control mice. The

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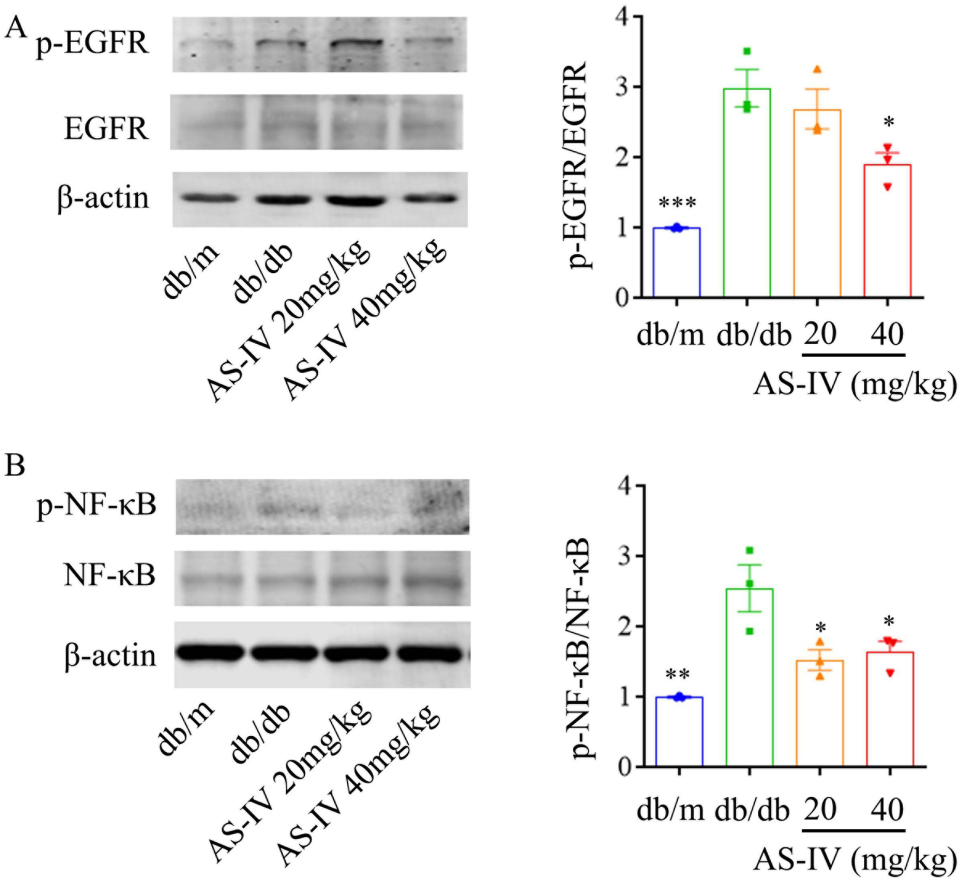

Figure 6 The effects of AS-IV on the EGFR/NF-κB pathway in db/db mice. Representative Western blot bands and grayscale analysis results for p-EGFR/EGFR (**A**), p-NF-KB/NF-KB (**B**). Values represented as means ± SEM (n = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the model group.

phosphorylation levels of EGFR (20 mg/kg AS-IV, 2.69 ± 0.49 versus 2.98 ± 0.46, n = 3; $p > 0.05$) (40 mg/kg AS-IV, 1.90 ± 0.29 versus 2.98 ± 0.46, n = 3; $p < 0.05$) and NF-κB (20 mg/kg AS-IV, 1.52 ± 0.25 versus 2.54 ± 0.58, n = 3; $p < 0.05$) (40 mg/kg AS-IV, 1.64 ± 0.26 versus 2.54 ± 0.58, n = 3; $p < 0.05$) in the hippocampal tissues of db/db mice were notably lowered following treatment with AS-IV at 20 mg/kg and 40 mg/kg. These findings indicate that AS-IV may improve cognitive function in db/db mice by influencing protein expression within the EGFR/NF-κB pathway.

Discussion

This research, utilizing two PET/CT techniques, revealed a significant increase in Aβ and tau protein accumulation in the hippocampus of older db/db mice, potentially causing diabetes-related cognitive decline. Notably, spatial learning and memory were enhanced in db/db mice after 8 weeks of AS-IV treatment, which may be associated with the TNF-α-activated EGFR/NF-κB pathway reducing Aβ and tau protein accumulation in the hippocampus.

Type DM2 is a complex physiological condition that impacts multiple organs, with a significant effect on the brain. DCI is a diabetes-induced alteration in the structure and function of the central nervous system, characterized by acquired cognitive impairment, vasculopathy and cranial nerve demyelination.[22] With the increasing prevalence of DM, the rate of cognitive impairment among elderly Chinese diabetic patients is as high as 48%.[23] Therefore, elucidating the underlying pathogenic mechanisms and developing innovative therapies are critical research priorities. Astragaloside IV, an active ingredient derived from Astragalus membranaceus, is capable of lowering fasting glucose levels and ameliorating many complications associated with DM.[24–26] In the present study, db/db mice deficient in leptin receptors developed marked increases in fasting blood glucose and body weight elevations, as well as impaired glucose tolerance at 8 weeks of age. In the administration groups, 8 weeks of oral gavage treatment in db/db mice led to different degrees of reduction in fasting

blood glucose, body weight gain, and glucose tolerance. It was found that db/db mice exhibit significant cognitive dysfunction as early as 7 weeks of age.[27] As expected, the gavage of AS-IV for 30 consecutive days significantly improves cognitive function in a rat model of DCI induced by streptozotocin (STZ) in combination with a high-fat, high-sugar diet, as evidenced by the MWM test.[28] In this study, db/db model mice demonstrated significant improvement in cognitive function after 8 weeks of AS-IV administration, which is consistent with previous studies. Research has shown a complex and significant association between blood glucose levels and cognitive performance. For people with diabetes, fluctuations in blood glucose levels appear to be strongly associated with several aspects of cognitive function, including attention, and visual perception, among others.[29] However, other studies have provided a different perspective, indicating that improvements in cognitive function may not rely on the regulatory state of peripheral blood glucose. For example, in a study of the Tg2576 mouse model of Alzheimer's disease, researchers found that improvements in cognitive function appeared to be more related to age-related differences in mechanisms than directly dependent on the regulatory state of peripheral blood glucose.[30] Thus, while improvements in peripheral blood glucose levels may be associated with enhanced cognitive function in some cases, the relationship is not always directly causal. Unlike acute administration, which is primarily used for rapid symptomatic relief, diseases such as DCI, which require long-term management, typically necessitate a chronic dosing strategy. The present study is in line with most of the previous studies focusing on DCI, which were concerned with observing the effects of chronic dosing. Studies have shown that the chronic administration of drugs can prevent complications, enhance therapeutic efficacy, and reduce side effects, thereby achieving a sustained and stable therapeutic effect.[31] In addition, chronic administration of drugs to experimental animals allows for a comprehensive assessment of their efficacy and safety profiles in the treatment of chronic diseases.[32] In this study, we administered AS-IV continuously to db/db mice and dynamically monitored its effects on body weight, blood glucose levels, and cognitive functions to investigate the pharmacodynamic properties of AS-IV. Based on this foundation, we aim to further investigate the underlying mechanisms of AS-IV in alleviating diabetes-related cognitive dysfunction.

The hippocampus, situated in the deep temporal lobe, plays an essential and complex role in various brain functions, including memory formation and spatial navigation. Maintaining the hippocampus's structural and functional integrity is critical for managing short-term memory, learning, executive skills, and attention, and for preventing cognitive decline.[33–35] Studies have shown that by combining different biomarkers, PET/CT imaging can assess the expression of relevant biomarkers in specific regions of the brain, which is important for distinguishing between different types of dementia.[36,37] Therefore, PET/CT imaging techniques were used in this study to observe the expression of Aβ and tau proteins in the mouse brain. The accumulation of Aβ is considered one of the key pathological features of AD and other forms of cognitive impairment. Studies have shown that Aβ deposition leads to structural changes in the brain and impaired clinical function.[38] Identified as a major link in AD progression, Aβ is associated with a higher rate of AD development among Aβ-positive older adults, suggesting that long-term Aβ accumulation elevates the chances of cognitive impairment and dementia in the elderly. Similarly, higher levels of serum Aβ could be involved in the development of cognitive impairment in type 2 diabetes mellitus.[39] This suggests that metabolic diseases may exacerbate cognitive impairment by affecting Aβ metabolism. In our prior investigation, we discovered the existence of Aβ plaques deposition in the hippocampus of rats with DE.[9] AS-IV activates PPARγ and inhibits BACE1 activity, thereby reducing Aβ generation and positioning it as a promising therapeutic strategy for neurodegeneration related to Aβ.[40] In addition, AS-IV has been discovered to enhance neuronal survival and functional recovery by adjusting the levels of neurotrophic factor and brain-derived neurotrophic factor.[41] In this study, we observed a significant increase in Aβ protein deposition in the hippocampal tissue of db/db mice using 18F-AV45 PET/CT. Further staining with Congo red revealed that the increased Aβ levels were mainly distributed in the DG, CA1, and CA3 regions of the hippocampus. Notably, AS-IV significantly reduced the deposition of Aβ plaques in the hippocampal tissue of db/db model mice, suggesting its potential therapeutic effects in DCI.

There is a complex interaction between Aβ protein deposition and tau protein accumulation. A longitudinal study using PET imaging suggests that tau protein accumulation tends to occur in brain regions affected by Aβ protein deposition, triggering a range of neurodegenerative processes that interact to promote the development of Lewy body dementia.[42] In the amyloid cascade hypothesis, tau protein is considered one of the main downstream targets of Aβ

protein and is associated with neurotoxicity.[43] PET imaging was used to assess tau protein levels, and a significant negative correlation was found between tau protein accumulation and neuropsychological scores.[44] In this study, we observed a marked increase in tau accumulation in the hippocampal tissue of db/db rats, as assessed by ^{18}F-MK6240 PET/CT. Notably, AS-IV treatment reversed this effect. As we known, in nerve cells, neurofibrillary tangles are formed by the aggregation of tau protein.[45] The results of glycine silver staining revealed that neurofibrillary tangles increased in the hippocampal tissue of db/db mice compared with db/m mice. Both AS-IV 20 mg/kg and 40 mg/kg treatments significantly reversed this process. The findings suggested that AS-IV decreased tau protein accumulation in the hippocampal tissues of db/db mice.

Diabetes can cause a systemic chronic inflammatory response.[46] Yang et al[47] discovered that TNF-α can reduce tight junction proteins, leading to blood-brain barrier disruption and activation of microglial and astrocytic cells in the CNS, resulting in brain inflammation. Serum levels of inflammatory factors such as TNF-α are strongly associated with cognitive impairment in T2DM patients.[48] ELISA assay showed that serum levels of TNF-α were elevated in db/db mice. Administering 20 mg/kg and 40 mg/kg of AS-IV led to a notable reduction in serum TNF-α levels in db/db mice. however, the levels did not completely revert to those seen in db/m mice. Immunofluorescence staining revealed that TNF-α expression was significantly elevated in the hippocampal DG, CA1, and CA3 regions of db/db mice. The process was significantly counteracted by AS-IV treatments of 20 mg/kg and 40 mg/kg. TNF-α transactivates the EGFR and exerts various immunomodulatory effects through the EGFR/NF-κB pathway.[49] The EGFR/NF-κB signaling pathway has been found to be involved in a variety of pathophysiological processes, such as cell proliferation, survival, differentiation, immune response, inflammatory response, and synaptic plasticity.[50] AS-IV protects cortical neurons from ischaemia/reperfusion injury by regulating EGFR activity.[14] Our results show a notable rise in the phosphorylation levels of EGFR and NF-κB in the hippocampal tissues of db/db model mice. After administering AS-IV at 20 mg/kg and 40 mg/kg, there was a marked reduction in the phosphorylation levels of EGFR and NF-κB in the hippocampal tissues of db/db mice. Further validation using pathway-specific inhibitors is required to establish the causal relationship between the EGFR/NF-κB pathway and the neuroprotective effects mediated by AS-IV.

However, this study had certain limitations. Firstly, since our study primarily observed the effects of AS-IV treatment only after 8 weeks, further research is needed to investigate the dynamic changes in cognitive function in diabetic db/db mice following AS-IV treatment. Secondly, The use of db/db model mice has limitations in terms of translation. While human DCI is typically associated with insulin resistance, chronic hyperglycemia, and inflammatory responses, the pathology of db/db model mice is primarily characterized by obesity and diabetes resulting from leptin receptor defects, which is not entirely consistent with human pathophysiological processes. In addition, the db/db model mice differ significantly from humans in both the expression and function of tau protein isozymes; thus, they cannot accurately mimic the pathological changes of human tau protein in diabetic cognitive impairment.[51] Thirdly, a comparison between AS-IV and other medications was not conducted, and there was a lack of combination interventions to further assess the impact of AS-IV on DCI. Consequently, further research is required to explore how AS-IV influences the TNF-α-activated EGFR/NF-κB pathway when other drugs are present in DCI.

Conclusion

In the present study, the PET/CT assay showed that AS-IV significantly reduced the deposition of Aβ and tau proteins in hippocampal tissue. The Morris water maze experiment further revealed that AS-IV significantly improved the cognitive ability of db/db mice. Further studies revealed that AS-IV may exert neuroprotective effects by modulating the TNF-α-activated EGFR/NF-κB signaling pathway. Future studies will confirm this mechanism of action through the use of EGFR/NF-κB pathway inhibitors. These results offer scientific support for AS-IV as a possible medication for treating DCI.

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Disclosure

The authors declare no conflict of interest in relation to this paper.

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