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ORIGINAL PAPER

Bexarotene Attenuates Focal Cerebral Ischemia–Reperfusion Injury via the Suppression of JNK/Caspase-3 Signaling Pathway

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Abstract

Apolipoprotein E (APOE) is implicated not only in chronic degenerative neurological diseases, such as Alzheimer's disease, but also in acute brain disorders, including traumatic brain injury. Bexarotene, a selective agonist of the retinoid X receptor, has been reported to enhance markedly the expression of APOE. Previous studies have indicated that bexarotene exerts neuroprotective effects in animal models of ischemic stroke by modulating the peripheral immune response and autophagy. However, the role of this drug in neuronal apoptosis and the potential mechanisms involved have yet to be elucidated. The present study employed transient middle cerebral artery occlusion (t-MCAO) as a model of acute cerebral ischemia/reperfusion injury. The experiments were performed in wild-type C57BL/6 mice and APOE gene knockout (APOE-KO) mice. After t-MCAO, mice received intraperitoneal injection of bexarotene (5 mg/kg) or an equal volume of the vehicle. The outcome measurements included neurological deficits, learning ability, spatial memory, infarct volume, histopathology, magnitude of apoptosis, and the level of expression of proteins of the JNK/caspase-3 signaling pathway. The obtained results demonstrated that bexarotene administration significantly improved neurological function, learning ability, and spatial memory in C57BL/6 mice, but not in APOE-KO mice. Infarct volume, tissue damage, neuronal apoptosis rate, and the expression of proteins involved in the JNK/caspase-3 signaling pathway were markedly increased after t-MCAO in both C57BL/6 and APOE-KO mice. Importantly, bexarotene treatment significantly ameliorated all these changes in C57BL/6, but not in APOE-KO mice. In conclusion, bexarotene markedly alleviates the neurological deficits, improves the histological outcome, and inhibits cell apoptosis in mice after t-MCAO. This effect is mediated, at least in part, by up-regulation of APOE. Thus, bexarotene may be a candidate drug for the treatment of cerebral ischemia patients.

Keywords Ischemia/reperfusion · Bexarotene · Apolipoprotein-E · Apoptosis · Neuroprotection

Introduction

Acute ischemic stroke is one of the most frequent cerebrovascular diseases and is characterized by high mortality and disability worldwide [1]. Prompt restoration of blood flow to the ischemic area in the appropriate time frame by pharmacological thrombolysis or mechanical thrombectomy is the most effective treatment [2, 3]. Although numerous neuroprotective agents have been investigated, few of them demonstrated definite clinical efficacy for the therapy of ischemic stroke. Therefore, an urgent need exists for the search for safe and effective medications for the treatment of this condition.

Apolipoprotein (APOE) is the major apolipoprotein maintaining lipid homeostasis in the human brain [4]. Previous studies have demonstrated that APOE is strongly

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linked to the progression and outcome of several neurological disorders. This association relies on the beneficial impact of APOE on the brain, including anti-inflammatory, anti-oxidative, anti-hypoxic, and anti-excitotoxic effects [5–7]. It has been documented that APOE-deficient mice display increased vulnerability to neuronal damage, and this effect may be attributed to the ability of APOE to regulate the expression of C/EBP homologous protein(CHOP) and proteins implicated in the c-Jun N-terminal kinase (JNK)-dependent apoptotic pathway [8].

Bexarotene, an agonist of the retinoid X receptor (RXR), is clinically approved drug for the treatment of refractory cutaneous T-cell lymphoma. Lately, bexarotene has been shown to enhance the clearance of soluble β-amyloid, improving neurological function in Alzheimer's disease mouse models, and this effect is APOE-dependent [9]. Subsequent studies documented multiple neuroprotective effects of bexarotene. This compound provides neuroprotection not only in chronic degenerative neurological diseases such as the Parkinson's disease [10] or amyotrophic lateral sclerosis [11], but also in acute brain injuries such as subarachnoid hemorrhage [12] and stroke [13]. Recently, bexarotene was shown to inhibits inflammatory response and cell apoptosis after traumatic brain injury in mice, improving the neurological function; this effect was mediated partially by APOE [14]. Moreover, we have previously demonstrated that bexarotene can decrease the permeability of the blood-brain barrier and reduce secondary brain edema in rats with cerebral ischemia/reperfusion (I/R) injury [4]. However, whether bexarotene protects the brain from I/R injury by affecting the expression of APOE remains unknown.

The present study addresses this question by analyzing the effects of bexarotene on the outcome of focal I/R injury in wild-type C57BL/6 and APOE-KO mice. The impact of bexarotene and the role of APOE was determined by evaluating the modified neurological severity score (mNSS), learning function, spatial memory, volume of cerebral infarct, hematoxylin-eosin(HE) staining of the hippocampal CA_1 region, cell apoptosis, and the expression of proteins involved in the JNK/caspase-3 signaling pathway.

Materials and Methods

Animals

Adult male C57BL/6 mice and APOE-KO mice on C57BL/6 genetic back ground, weighing 20–25 g, were obtained from the Animal Laboratory Center of Chongqing Medical University (Chongqing, China). Mice were housed in a temperature-controlled (25 ± 1 °C) facility with a 12-h light-dark cycle and maintained on standard food and water available adlibitum. All animal experiments were in accordance with the guidelines of the National Institutes of Health and were approved by the Experimental Ethics Committee of Chongqing Medical University (License Number: SYXK YU 2010-001).

Preparation of Cerebral Ischemia–Reperfusion Model and Drug Administration

The cerebral ischemia–reperfusion model was induced by transient middle cerebral artery occlusion (t-MCAO) lasting for 60 min, as previously described [15]. Briefly, animals were anesthetized with 3% isoflurane in a 67%/30% mixture of N_2O/O_2, and 1.5% of isoflurane was present during the surgery to maintain the anesthesia. Under an operating microscope, a small incision was made on the surface of the neck. The right common carotid artery, the external carotid artery, and the internal carotid artery were exposed. Subsequently, the right common carotid artery and the external carotid artery were ligated with a 3-0 silk suture. The internal carotid artery was temporarily occluded with a microvascular clip. A small "V" shaped incision was performed near the bifurcation of the right common carotid artery. A silicone-coated nylon filament was inserted into the internal carotid artery through the incision and advanced 10–12 mm distal to the carotid bifurcation, to reach the point of origin of the middle cerebral artery. The nylon filament was taken out after 60 min of occlusion to allow reperfusion. Sham-operated mice underwent the same surgical procedures except for the occlusion of the middle cerebral artery. Throughout the surgical procedure, the body temperature was maintained at 36.5 ± 0.5 °C using a heating lamp until the mice completely recovered from the anesthesia.

70 male C57BL/6 mice and 60 male APOE-KO mice were randomly separated into the following experimental groups: (1) sham surgery, (2) vehicle-operated I/R (vehicle) group (3) bexarotene-treated I/R (bexarotene) group. The solution of bexarotene (#ab141025, Abcam, Cambridge,UK) was prepared as previously described [14, 16]. The bexarotene, 5 mg/kg, or the vehicle were administered by intraperitoneal injection immediately after t-MCAO (60 min), and the injections were repeated daily injection until were sacrificed.

Behavioral Testing

A series of behavioral tests were performed before t-MCAO and 1, 3, 7 and 14 days after the procedure two investigators blinded to the type of treatment. The neurologic deficit evaluation was performed by the mNSS test as previously described [17]. The result of the mNSS test is a composite of general, focal, balance, reflex, and sensory tests with a minimum of 0 points in normal mice to a maximum of 18 points in severely injured mice [18, 19].

For the rotarod test, mice were placed on an accelerating rotarod cylinder, and the rotating speed was increased from 4 to 40 rpm with in 5 min [20]. The time for which the mice stayed on the rotarod was recorded. The trial ended when the mice fell off the rungs or gripped the rod and spun around for two revolutions without attempting to walk on the rod. The mice were trained to perform this protocol three times per day for 3 consecutive days before t-MCAO, and the final average score on the last day before the surgery was considered the baseline.

The spatial learning and memory ability of the mice were determined by the Morris water maze (MWM) as previously described [21, 22]. The mice were trained before t-MCAO and tested from 16 to 21 days after the surgery. During each trial, mice were made to swim freely in a pool in which a hidden platform was placed 1 cm under the water surface. The mice had 90 seconds to find the platform; if they did not reach the platform within the allotted time, they were guided to it and allowed by the researchers to stay on it for 5 s. On the sixth day, the platform was removed, the mice were released into the pool from the site opposite to the previous location of the platform, and the swimming track and the number of times the site of the platform was crossed were recorded.

Measurement of Infarction Volume

Infarct volume was quantified by 2, 3, 7-triphenyltetrazolium chloride (TTC) staining as described previously [17, 23]. Briefly, 24 h after t-MCAO the brains were quickly isolated, frozen, and sliced into 2 mm thick consecutive coronal slices. The slices were immersed into 2% TTC solution for 15 min at 37 °C and fixed with 4% paraformaldehyde at 4 °Covernight. Images of the slices were captured by a digital scanner, and the infarct volume was calculated using Image-Pro Plus6software (Media Cybernetics, Rockville, MD, USA).

Magnetic Resonance Imaging

Infarct volume at 3 days after t-MCAO was measured by multi-slice T2-weighted magnetic resonance imaging (MRI) performed with aBruker 7T (70/20) system (BrukerBiospin, Billerica, MA, USA) [20]. Isoflurane-anesthetized mice were placed inside the magnet equipped with a quadrature volume coil. Images were acquired using repetition time of 3000 ms, echo timeof 25 ms, image size of 128*128 pixels, field of view 2.5 cm, slice thickness of 0.8 mm. Together, 20 slices were obtained, and the MRI images were analyzed using Bruker Para Vision 6.0 software.

Western Blotting

At 1, 3, and 5 days after t-MCAO, the C57BL/6 mice and APOE-KO mice from the sham-operated group, vehicle group, and bexarotene group were deeply anesthetized, and the brains were rapidly dissected. The ischemic brain tissue was washed with phosphate-buffered saline (PBS) and subsequently transferred to a 1.5 ml centrifuge tube with RAPI lysis buffer (P00113D; Beyotime, Shanghai, China) that contained proteinase and phosphatase inhibitor cocktail. The homogenates were centrifuged at 12,000×*g* for 5 min at 4 °C and the supernatants were collected as total proteins. The protein samples were quantified with a bicinchoninic acid protein assay kit (P0012S, Beyotime, China). An 8 μL sample of the protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membranes were blocked for 1 h with 5% BSA at room temperature and then incubated with specific primary antibodies: rabbit monoclonal anti-JNK (#9252, Cell Signaling Technology, 1:1000), rabbit monoclonal anti-phospho-JNK (1/2) (#4668, Cell Signaling Technology, 1:1000), rabbit monoclonal anti-phospho-C-JUN (#3270, Cell Signaling Technology, 1:1000), rabbit monoclonal anti-caspase-3(#14220, Cell Signaling Technology, 1:1000), rabbit monoclonal anti-APOE (#13366, Cell Signaling Technology, 1:1000), rabbit polyclonal anti-cleaved caspase-3 (#ab49822, Abcam, 1:1000), and β-actin (#20536-1-AP, Proteintech, 1:3000). Subsequently, the membranes were washed three times in TBST and incubated with HRP-conjugated secondary antibodies (#SA00001-2, Proteintech, 1:5000) for 1 h at room temperature. Following three washes in TBST, immunoreactive bands were detected using the enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA).

HE Staining

Three days after t-MCAO, mice from each group(n = 3)were deeply anesthetized and transcardially perfused with heparinized saline followed by 4% neutral-buffered paraformaldehyde. Brains were removed and fixed with the same solution for 24 h. Subsequently, the brain tissue was dehydrated in gradient concentrations of ethanol and xylene, sliced into 5-μm-thick sections and stained with HE. Three to five sections with typical hippocampus changes in each animal were photographed under light microscopy.

Immunofluorescence Staining

For immunofluorescence staining, the tissue was prepared as described above for the HE staining, but the duration of fixation was shortened to 12 h. Frozen sections of the brain,

5 μm thick, in the coronal plane were prepared and incubated at 4 °C overnight with the rabbit anti-cleaved caspase-3 polyclonal antibody (#ab49822, Abcam, 1:250) diluted 1:100. Subsequently, the sections were incubated with a fluorescent secondary antibody (DyLight 594, goat anti-rabbit IgG, Abbkine, California, USA) at 37 °C for 1 h. The sections were analyzed using a fluorescence microscope(Eclipse Ti-S; Nikon, Tokyo, Japan). The integrated optical density (IOD) of cleaved caspase-3 staining was calculated using the Image-Pro Plus 6 software.

TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated dUTP-biotinnick-end labeling (TUNEL) staining was employed to assess cell apoptosis in the cortex. On day 3 post t-MCAO, mice from each group (n = 3) were disposed with the same procedures as in the "immunofluorescent staining". The 5 μm thick frozen sections were stained using the In Situ Cell Death Detection Kit (Vazyme Biotech Co, Ltd, Nanjing, China) according to the manufacturer's instructions. Nuclei were stained with DAPI (C1006, Beyotime). The bright red-labeled cell nuclei were considered TUNEL-positive cells. Three to four sections in each animal were photographed by a fluorescence microscope and analyzed using the Image-Pro Plus 6 software.

Statistical Analysis

The results are expressed as the mean ± standard deviation (SD). Statistical analyses were performed with the SPSS 17.0 software (IBM Corp, Chicago, IL, USA).The data of the mNSS, rotarod, and MWM tests, with the genotype and the type of treatment as the independent variables, were analyzed by a two-way analysis of variance(ANOVA), followed by the Tukey's test across the groups. The remaining data were evaluated by one-way ANOVA followed by the Tukey's test. P values < 0.05 were considered to represent statistical significance.

Results

Bexarotene Improved Neurological Outcomes Partially by the Expression of APOE

mNSS

In both C57BL/6 and APOE-KO mice, the t-MCAO injury resulted in a dramatic increase in mNSS scores in comparison with the respective sham-operated group($P < 0.05$; Fig. 1A). InC57BL/6 mice, treatment with bexarotene significantly decreased the mNSS scores at 3, 7, and 14 days after t-MCAO ($P < 0.05$; Fig. 1A-a). For APOE-KO mice, the treatment with bexarotene also decreased the mNSS scores after t-MCAO, but this changes did not reach statistical significance(Fig. 1A-b). In addition, the mNSS was significantly higher in the bexarotene group in APOE-KO mice on days 3, 7, and 14 after t-MCAO compared with the bexarotene group in C57BL/6 mice ($P < 0.05$; Fig. 1A-c).

Rotarod Test

In both C57BL/6 and APOE-KO mice, the I/R injury reduced the time on the rotating rod in comparison with the respective sham-operated groups ($P < 0.05$; Fig. 1B). For the C57BL/6 mice, treatment with bexarotene increased the time spent on the rod at 1, 3, 7, and 14 days after t-MCAO. The administration of bexarotene to the APOE-KO mice increased the time only at 7 and 14 days post-t-MCAO, but these changes were not statistically significant (Fig. 1B-a, b). Moreover, the duration in the bexarotene group of C57BL/6 was not significantly longer compared with that in the bexarotene group of APOE-KO mice(Fig. 1B-c).

Morris Water Maze (MWM) Test

Learning and spatial memory functions were evaluated using the MWM test. Both C57BL/6 and APOE-KO mice subjected to t-MCAO exhibited a longer latency than sham-operated animals ($P < 0.05$; Fig. 2). The treatment of C57BL/6 mice with bexarotene of significantly reduced the latency to escape at 16 and 19 days after the injury, as compared to the group treated with the vehicle only ($P < 0.05$; Fig. 2A-a). The administration of the drug reduced also the latency at days 16 and 19 post-t-MCAO in APOE-KO mice, but this decrease was not statistically significant ($P > 0.05$; Fig. 2B-a). The hidden platform was removed at day 20 after t-MCAO, and the swimming track and the number of times the platform had been crossed were recorded. In C57BL/6 group, mice treated with the vehicle showed fewer times to cross the platform than did the sham-operated mice, while the treatment with bexarotene significantly increased the number of crossings ($P < 0.05$; Fig. 2A-b). In the APOE-KO group, vehicle-treated mice with t-MCAO injury crossed the platform fewer times than sham-operated mice. Although the treatment with bexarotene increased the number of crossings, this difference was not statistically significant ($P > 0.05$; Fig. 2B-b). Moreover, the bexarotene group in C57BL/6 mice had significantly shorterlatency, more times to pass over compared with the bexarotene group in APOE-KO mice ($P < 0.05$; Fig. 2C-a,b). These results demonstrated that bexarotene administration significantly improved the learning and spatial memory functions of C57BL/6 mice after t-MCAO, but not of APOE-KO mice.

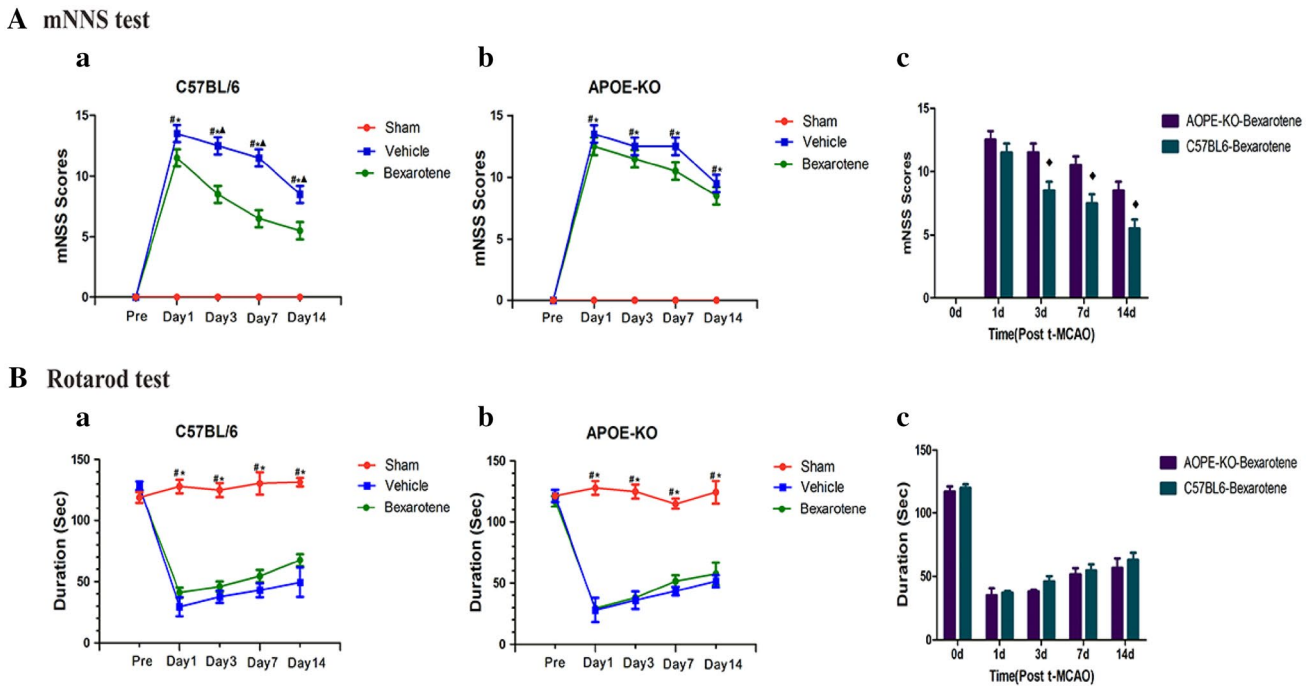

Fig. 1 Bexarotene improved neurological outcomes after t-MCAO. The mNSS of C57BL/6 (**A, a**) and APOE-KO mice (**A, b**) and the duration of the rotarod test of C57BL/6 (**B, a**) and APOE-KO mice (**B, b**) were examined prior to injury, and 1, 3, 7, and 14 days after t-MCAO. The comparison of mNSS (**A, c**)and duration(**B, c**) between the bexarotene groups in C57BL/6 and APOE-KO mice respectively. (n = 8 per group; $^{\#}P < 0.05$ sham vs. vehicle, $^{\star}P < 0.05$ sham vs. bexarotene, $^{\blacktriangle}P < 0.05$ bexarotene vs. vehicle, $^{\blacklozenge}P < 0.05$ bexarotene of C57BL/6 vs. bexarotene of APOE-KO) (Color figure online)

Bexarotene Increased APOE Expression in Wild-Type C57/BL6 Mice

To determine whether bexarotene injected intraperitoneally can reach the site of injury and improve the expression of APOE in wild-type C57/BL6 mice, the expression of the target protein in mice treated with bexarotene and vehicle was analyzed by Western blotting at 1, 3, and 5 days after the I/R injury. In comparison with the vehicle group, the administration of bexarotene progressively increased the expression of APOE from day 1 to day 5 post t-MCAO ($P < 0.05$; Fig. 3).

Effects of Bexarotene on Infarction Volume

TTC Staining

To evaluate the effect of bexarotene on focal ischemia-induced brain injury, consecutive brain sections were stained with TTC 1 day after t-MCAO (Fig. 4A). In comparison with the vehicle group, bexarotene treatment of C57/BL6 mice markedly decreased the volume of the infarct ($P < 0.05$; Fig. 4A-b). In contrast, in APOE-KO mice, the difference between bexarotene and vehicle treated was not statistically significant ($P > 0.05$; Fig. 4A-c). In addition, the volume of the infarct was significantly less in the bexarotene group of C57BL/6 mice compared to the bexarotene group of APOE-KO mice ($P < 0.05$; Fig. 4A-d). These results indicate that the bexarotene treatment significantly reduces I/R-induced the ischemic area in the brain of wild-type C57/BL6 mice.

MRI Imaging

Multi-slice T2-weighted MRI with a Bruker 7T system was used to evaluate the volume of I/R-induced lesion at 3 days following t-MCAO. Figure 4B illustrates representative T2 images of the entire t-MCAO lesions in bexarotene treated and vehicle-treated mice (Fig. 4B). The lesions are represented by the hyperintense areas. In comparison with vehicle-treated animals, the lesion volumes in the bexarotene-treated C57/BL6 mice were significantly decreased ($P < 0.05$; Fig. 4B-a,b). Conversely, bexarotene intervention did not affect the lesion volume in APOE-KO mice ($P > 0.05$; Fig. 4B-c,d). These results demonstrate that bexarotene treatment reduced the volume of vasogenic edema and lesion caused by t-MCAO in wild-type C57/BL6 mice. In addition, the volume of vasogenic edema and lesion was significantly less in the bexarotene group of C57BL/6 mice compared to the bexarotene group of APOE-KO mice($P < 0.05$; Fig. 4B-e).

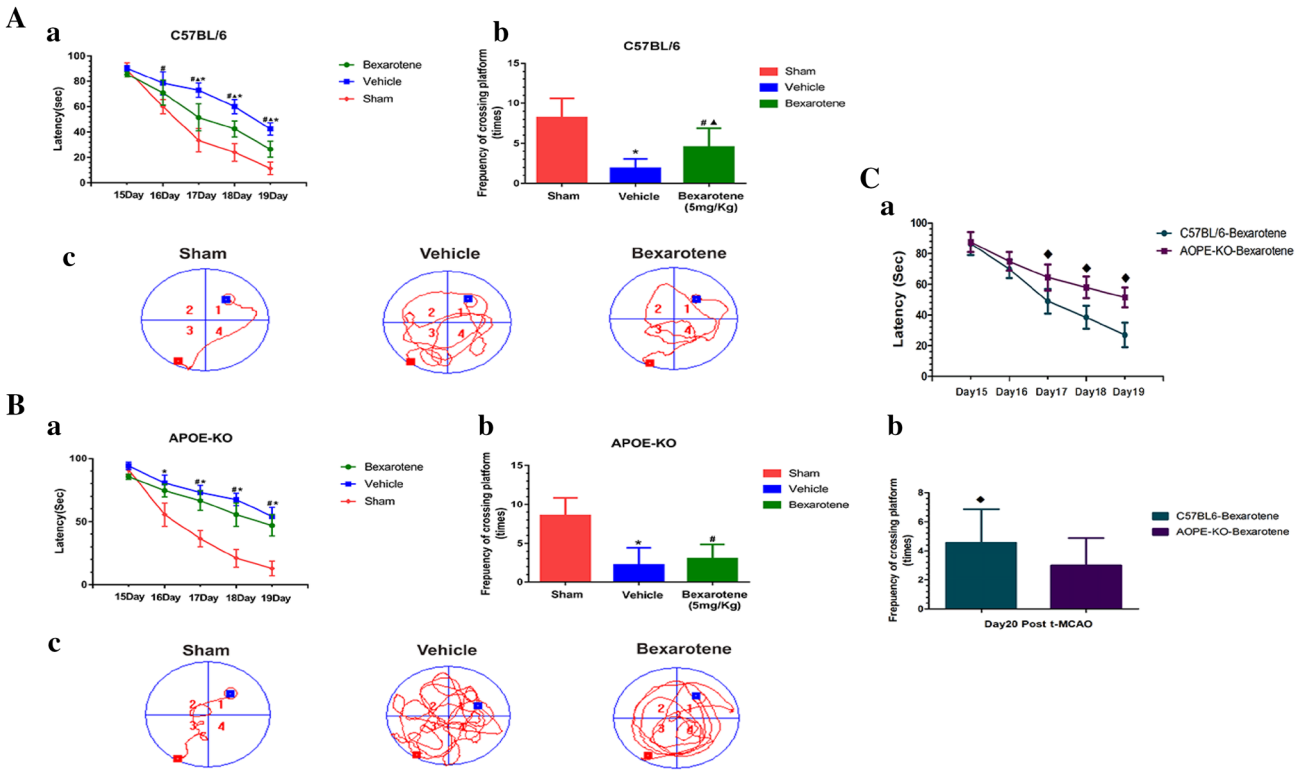

Fig. 2 Effects of bexarotene on learning and memory in C57BL/6 and APOE-KO mice based on the MWM test. The latency to locate the hidden platform in C57BL/6 mice (**A**, **a**) and APOE-KO mice (**B**, **a**) on day 15 and 19 after t-MCAO. The number of times the platform was crossed by C57BL/6 (**A**, **b**) and APOE-KO (**B**, **b**) mice 20 days after t-MCAO. The path before landing on the hidden platform by C57BL/6 (**A**, **c**) and APOE-KO (**B**, **c**) mice. **C**, **a**, **b** are the comparison of latency and times crossed the platform between the bexarotene groups in C57BL/6 and APOE-KO mice respectively. (n = 8 per group; $^{\#}P < 0.05$ sham vs. vehicle, $^{\star}P < 0.05$ sham vs. bexarotene, $^{\blacktriangle}P < 0.05$ bexarotene vs. vehicle, $^{\blacklozenge}P < 0.05$ bexarotene of C57BL/6 vs. bexarotene of APOE-KO) (Color figure online)

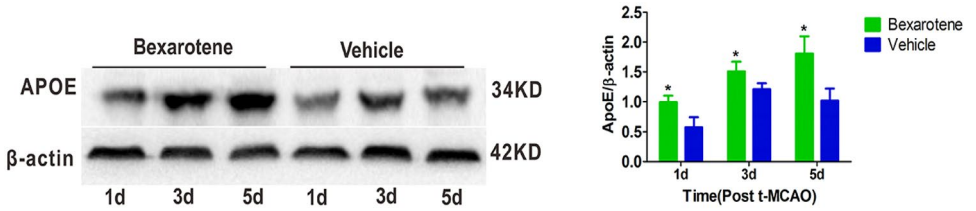

Fig. 3 Expression of APOE after bexarotene administration. Western blot analysis of APOE protein expression levels in the C57BL/6 mice at 1, 3, and 5 days after t-MCAO. Histograms show the quantification of APOE expression normalized to β-actin. Data are expressed as mean ± SD (n = 3 per group; $^{\star}P < 0.05$ bexarotene vs. vehicle) (Color figure online)

Effects of Bexarotene on Neuronal Injury

HE-stained sections were analyzed to determine the presence of necrotic cells in the hippocampal CA1 region of the brain subjected to I/R. As shown in Fig. 5, hippocampal CA1 region neurons in the sham-operated C57BL/6 and APOE-KO mice were arranged in regular rows, displayed intact cell structure, and had a clear outline of the nucleus. After t-MCAO, the neurons were necrotic with marked shrinking, cellular edema, and nuclear condensation. In the C57/BL6 mice, bexarotene treatment significantly prevented neuronal cell damage and improved the pathology associated with I/R injury (Fig. 5A, C). However, in the bexarotene-treated APOE-KO mice, the morphology of neurons also had been improved to some extent (Fig. 5B, D), but the improvement was not significant in comparison with C57BL/6 mice. Moreover, the grade of neuronal injury was significantly higher in the bexarotene group of APOE-KO mice compared to the bexarotene group of C57BL/6 mice ($P < 0.05$; Fig. 5E)

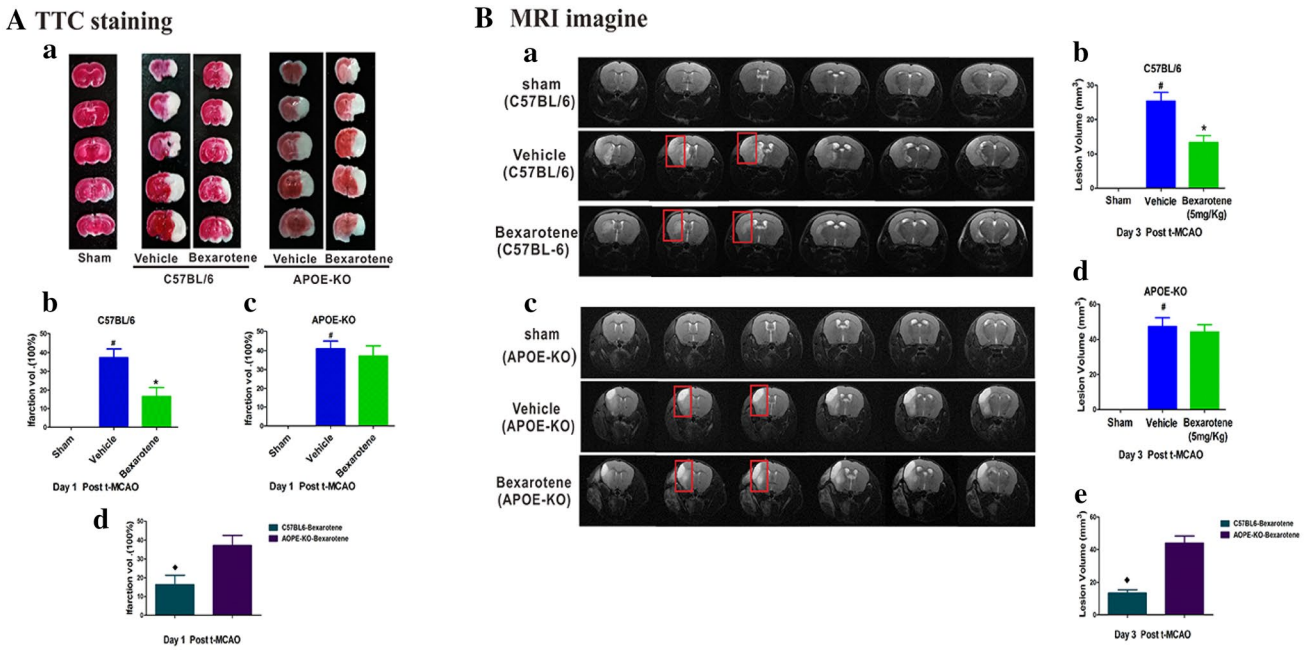

Fig. 4 Bexarotene reduced the volume of the t-MCAO induced infarct. **A**, **a** Representative maps of TTC-stained brain sections of different groups. **A**, **b** and **A**, **c** depict the volume of brain infarct in C57/BL6 and APOE-KO mice calculated as the fraction of the hemisphere. Data are expressed as mean ± SD (n = 3 per group; $^{\#}P < 0.05$ sham vs. vehicle, $^{\star}P < 0.05$ bexarotene vs. vehicle). **B**, **a** and **B**, **c** Representative images of T_2-weighted MRI show the entire lesion volume in the brain of vehicle-treated and bexarotene-treated C57/BL6 and APOE-KO mice at 3 days after t-MCAO. **B**, **b** and **B**, **d** show the volume of brain infarct in C57/BL6 and APOE-KO mice calculated as the fraction of the hemisphere. **A**, **d** and **B**, **e** are the comparison of volume of brain infarct between the bexarotene groups in C57BL/6 and APOE-KO mice. (n = 3 per group; $^{\#}P < 0.05$ sham vs. vehicle, $^{\star}P < 0.05$ bexarotene vs. vehicle, $^{\blacklozenge}P < 0.05$ bexarotene of C57BL/6 vs. bexarotene of APOE-KO) (Color figure online)

Effects of Bexarotene on Cell Apoptosis

TUNEL Staining

TUNEL staining was performed to evaluate the I/R-induced cell death in the cerebral cortex 3 days after t-MCAO. The red color indicated positive TUNEL staining, as shown in Fig. 6. In comparison with sham-operated mice, the number of TUNEL-positive cells significantly increased in vehicle- and bexarotene-treated C57BL/6 and APOE-KO groups. The bexarotene treatment significantly decreased the number of TUNEL-stained cells in C57BL/6 mice ($P < 0.05$; Fig. 6A, C). A small decrease in TUNEL-stained cells seen in bexarotene-treated APOE-KO mice at 3 days after t-MCAO with did not reach statistical significance ($P > 0.05$; Fig. 6B, D). In addition, the number of TUNEL-positive cells was significantly less in the bexarotene group of C57BL/6 mice compared to the bexarotene group of APOE-KO mice ($P < 0.05$; Fig. 6E).

Cleaved Caspase-3 Staining

Cleaved caspase-3 plays an essential role in the process of cell apoptosis. Western blotting revealed that bexarotene treatment markedly decreased the level of cleaved caspase-3 in C57BL/6 mice after I/R injury. On this basis, the presence of cleaved caspase-3-positive cells was determined by immune fluorescence on at 3 days following t-MCAO. The positive signal was indicated by red staining of the cells. In comparison with sham-operated group, the IOD of cleaved caspase-3 positive staining was significantly increased in the vehicle-and bexarotene-treated C57BL/6 and APOE-KO mice (Fig. 7). In C57BL/6 mice, the administration of bexarotene significantly decreased the IOD of the positive staining ($p < 0.05$; Fig. 7A, C). Conversely, in APOE-KO mice this difference was not statistically significant ($P > 0.05$; Fig. 7B, D). In addition, the IOD of cleaved caspase-3 positive staining was significantly less in the bexarotene group of C57BL/6 mice compared to the bexarotene group of APOE-KO mice ($P < 0.05$; Fig. 7E).These results indicate that cell apoptosis rate was lower in C57BL/6 mice compared to APOE-KO mice.

Effects of Bexarotene on Protein Expression of JNK/Caspase-3 Signaling Pathway

To determine the effects of bexarotene on the t-MCAO-induced changes in JNK/caspase-3 signaling pathway in

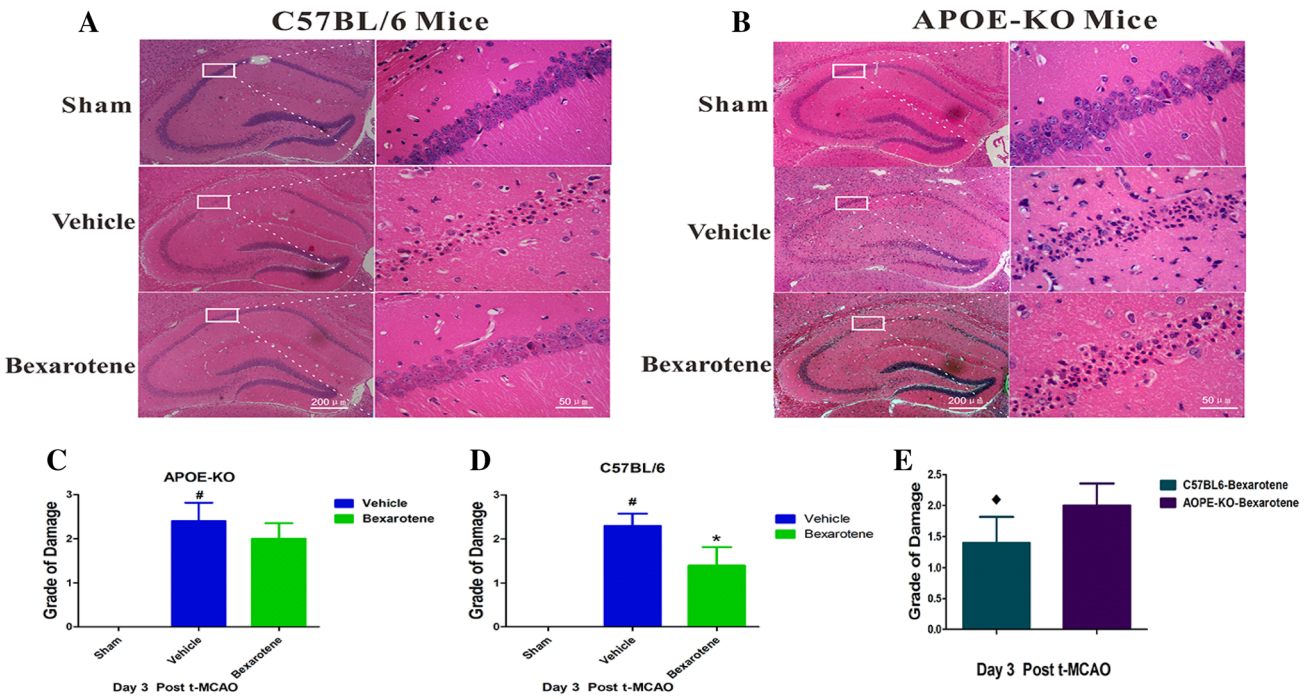

Fig. 5 Images of HE-stained sections of the hippocampal CA1 region in C57BL/6 (**A**) and APOE-KO (**B**) mice at 3 days after t-MCAO. Quantitative analysis is shown in **C** and **D**. **E** is the comparison of the grade of neuronal injury between the bexarotene groups in C57BL/6 and APOE-KO mice. (n = 3 per group; $^{\#}P < 0.05$ sham vs. vehicle, $^{\star}P < 0.05$ bexarotene vs. vehicle, $^{\blacklozenge}P < 0.05$ bexarotene of C57BL/6 vs. bexarotene of APOE-KO) (Color figure online)

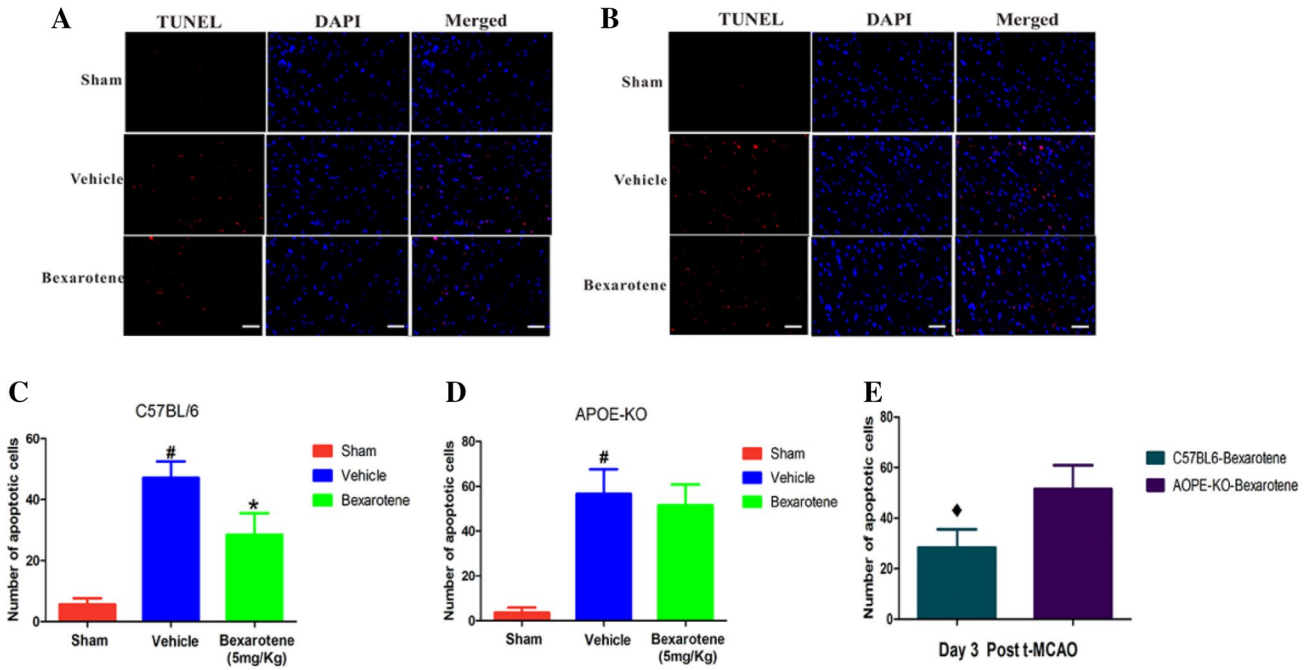

Fig. 6 Bexarotene inhibited cell apoptosis after t-MCAO. **A**, **B** Representative images of TUNEL-positive cells (red nuclei) in the cerebral cortex region of C57BL/6 (**A**) and APOE-KO (**B**) mice at day 3 after t-MCAO. Scale bar, 50 μm. **C**, **D** The number of TUNEL-positive cells in C57BL/6 and APOE-KO mice. **E** is the comparison of the number of TUNEL-positive cells between the bexarotene groups in C57BL/6 and APOE-KO mice. Data are expressed as mean ± SD (n = 3 per group; $^{\#}P < 0.05$ sham vs. vehicle; $^{\star}P < 0.05$ bexarotene vs. vehicle; $^{\blacklozenge}P < 0.05$ bexarotene of C57BL/6 vs. bexarotene of APOE-KO) (Color figure online)

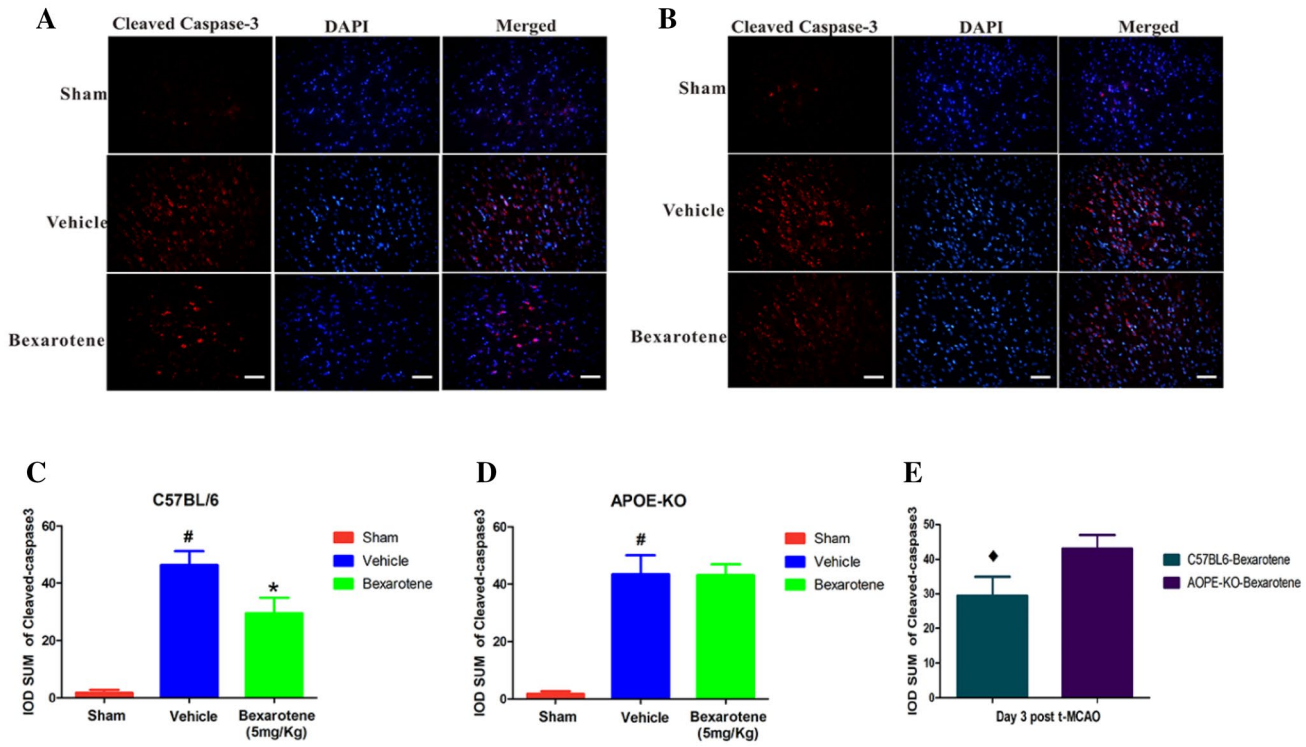

Fig. 7 **A**, **B** Representative immunofluorescence staining of cleaved caspase-3 (red) in the cerebral cortex region of C57BL/6 and APOE-KO mice at 3 days after t-MCAO respectively. Scale bar, 50 μm. **C**, **D** Histograms showing the IOD of cleaved caspase-3 positive staining in C57BL/6 and APOE-KO mice. **E** is the comparison of the IOD of cleaved caspase-3 positive staining between the bexarotene groups in C57BL/6 and APOE-KO mice. Data are expressed as mean ± SD (n = 3 per group; $^{\#}P < 0.05$ sham vs. vehicle; $^{\star}P < 0.05$ bexarotene vs. vehicle; $^{\blacklozenge}P < 0.05$ bexarotene of C57BL/6 vs. bexarotene of APOE-KO) (Color figure online)

proximity of the injury site, the expression of P-Jnk, Jnk, P-C-jun, caspase-3 and cleaved caspase-3 in the brain of wild-type C57BL/6 and APOE-KO mice were measured by Western blotting at 1, 3, and 5 days after t-MCAO. This analysis demonstrated that in comparison with the sham-operated group, the expression of P-Jnk, P-C-jun, and cleaved caspase-3was significantly increased after t-MCAO in a time-dependent manner in both wild-type C57BL/6 and APOE-KO mice (Fig. 8A, B). Bexarotene administration significantly prevented these increases in C57BL/6 mice ($P < 0.05$; Fig. 8A-b, c, d), but not in APOE-KO mice ($P > 0.05$; Fig. 8B-b, c, d), These findings indicate that bexarotene exerts an anti-apoptotic effect via the JNK/caspase3 signaling pathway, and this effect is partially dependent on the expression of APOE.

Discussion

Ischemic stroke accounts for nearly 86% of cases of stroke [1]. Currently, recanalization by thrombolysis or mechanical thrombectomy is the primary therapeutic strategy for the restoration of blood supply. However, reperfusion of the ischemic tissue is often accompanied by inflammation, oxidative stress, calcium overload, apoptosis and necrosis, and these events aggravate the cerebral microcirculation and disturb cellular homeostasis, and may lead to the death of neurons [17, 23]. Therefore, the search for agents effectively improving the outcome of ischemic stroke is an urgent clinical necessity.

Bexarotene is an FDA-approved drug for the treatment of cutaneous lymphoma and has also been shown to be protective in a wide variety of brain diseases [24]. Previous studies have demonstrated that bexarotene markedly improves the neurological function of mice after TBI, and this effect is mediated in part by up-regulation of APOE [14]. In addition, bexarotene also exhibited a protective effect in stroke models by modulating the peripheral immune response [13]. In both studies, a relatively low dose (5 mg/kg and 0.5–25 mg/kg, respectively) was used. This dose of the drug is lower than that used to treat cancer and produced no apparent side effects. The present study utilized bexarotene at 5 mg/kg and demonstrated that this compound significantly improves the neurological outcomes after I/R injury, including the reduction of mNSS, the extension of the time on the rotating rod, and the improvement of the spatial memory. Moreover, the obtained results indicate that bexarotene treatment significantly decreases the volume of ischemic lesion

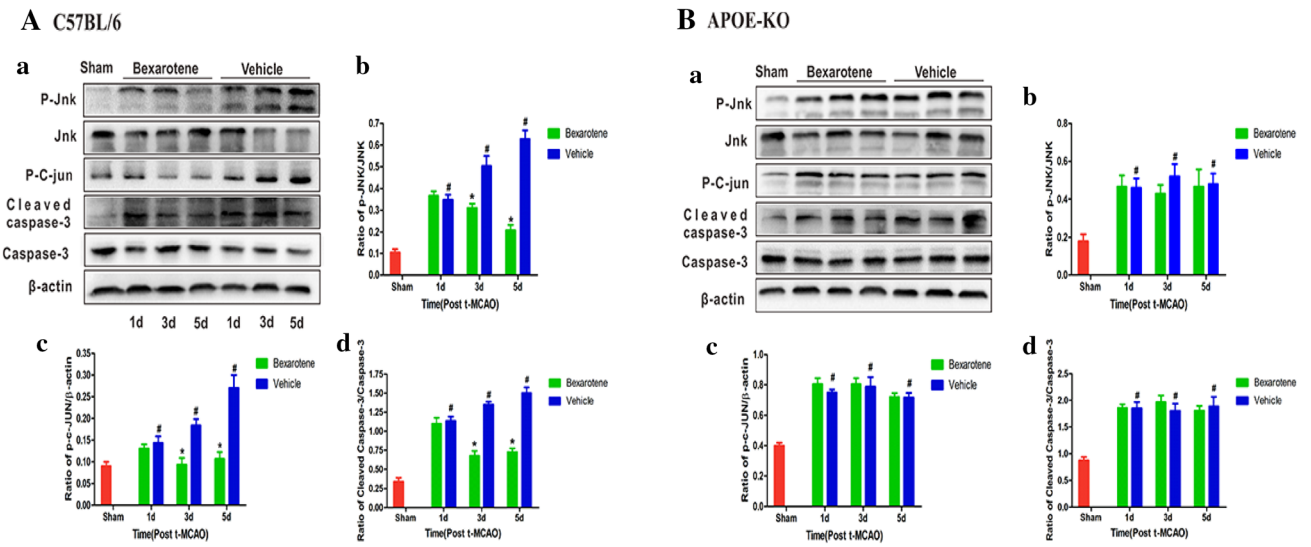

Fig. 8 Bexarotene inhibited the activation of JNK/Caspase-3 signaling. **A**, **a** and **B**, **a** Time-dependent expression of P-Jnk, Jnk, P-C-jun, caspase-3, and cleaved caspase-3 in C57BL/6 and APOE-KO mice at 1, 3, and 5 days after t-MCAO determined by Western blotting. **A**, **B** panels **c, b** and **d** presenting quantification of P-C-jun expression normalized to β-actin, ratio of phosphorylated to total Jnk, and caspase-3 in C57BL/6 and APOE-KO mice respectively. Data are expressed as mean ± SD. (n = 3 per group; $^{\#}P < 0.05$ sham vs. vehicle, $^{\star}P < 0.05$ bexarotene vs. vehicle) (Color figure online)

volume in mice after t-MCAO. The relevance of these findings is underscored by the fact that either a better neurological outcome or a smaller infarct volume are considered to represent critical indicators for stroke patients[25–27]. Of note, neuronal apoptosis, synaptic structural damage, and neuroinflammatory response are the main causes of motor deficits after stroke [28, 29]. A recent study has shown that long-term administration of bexarotene restores the age-dependent loss of synaptic proteins by acting on a neural LRP1-APOE-meditated pathway but is accompanied by serious adverse events such as glial cell activation and body weight loss [30]. Another report provided data indicating that treatment of APOE4-deficient mice with bexarotene reverses the APOE4-induced cognitive and neuronal impairment [31]. Moreover, significant behavioral differences were detected between aged APOE-KO mice and age-matched wild type mice; except for the rotarod test, the APOE-KO mice exhibited clear deficits in learning and memory [32]. In agreement with those results, the present research demonstrates that bexarotene significantly improves neurological outcomes in wild-type C57BL/6 but not in APOE-KO mice, except for the rotarod test. The possible mechanism of this outcome is up-regulation of APOE expression by bexarotene. Thus, a relatively lower dose and short-term administration of bexarotene may be a feasible therapeutic approach for acute brain injury, such as an ischemic stroke.

APOE is a pleiotropic molecule exhibiting three common polymorphisms in the APOE gene, with the ε2, ε3, and ε4 genes encoding three protein isoforms, APOE2, APOE3, and APOE4 respectively [6]. Recent evidence has indicated that responses to the treatment of neurological diseases may differ according to the APOE genotype. The APOEε4 gene is associated with increased risk for AD [6], AD with cerebral amyloid angiopathy, and TBI [6], while in AD patients APOEε2 appears to decrease this risk [6]. A meta-analysis reported that the APOEε2 allele frequency is associated with the risk of Parkinson's disease, while no susceptibility associated with the APOE ε3 or ε4 alleles was found [6]. In patients with an acute ischemic stroke, a series of studies assessing large cohorts of different ethnicities revealed the absence of correlation between the genotype of APOE and the clinical outcome [6].

Previous studies have demonstrated that APOE and its mimetic peptide analogs, such as COG1410, exert neuroprotective role in the central nervous system by anti-inflammatory and anti-oxidative activity [6]. Extensive research has revealed that APOE-deficient mice exhibit more severe motor and behavioral deficits in comparison with the wild-type mice [33]. The potential explanation for this difference may be that the deficiency of APOE aggravates cerebral edema by increasing the permeability of the blood-brain barrier (BBB), exacerbating histopathological damage [34]. Our results are in agreement with recent reports suggesting that bexarotene treatment significantly reduces lesion volume and improves histopathological recovery after I/R injury in wild-type C57/BL6 but not in APOE-KO mice.

The c-Jun N-terminal kinase (JNK) is an important member of the mitogen-activated protein kinase family. JNK

regulates various processes such as brain repair, memory formation, neuronal apoptosis, and neuroinflammation [35]. The current study documented that bexarotene markedly attenuated the phosphorylation of JNK and, as a result, down-regulated the expression of P-C-jun protein in wild-type C57/BL6 mice but not in APOE-KO mice. This finding indicates that bexarotene is strongly associated with JNK signaling pathway by the expression of APOE. Several other studies have also shown that APOE can affect JNK signaling pathway. Yang and collaborators have reported that down-regulation of the low-density lipoprotein receptor-related protein 1 (LRP1), which is a major receptor for APOE leads to the activation of the JNK signaling pathway[36]. This process may be mediated by the C-terminal fragment of LRP1which recruits JNK-interacting proteins and modulates the activation of JNK [37]. Another study has demonstrated that the interaction between APOE and lipoprotein receptors results in decreased activation of JNK, and c-secretase activity and G proteins participate in this effect [38]. However, the precise mechanism by which APOE affects JNK remains to be identified and will be the focus of our future work.

The pathogenesis of cerebral I/R injury involves diverse mechanisms, but increasing evidence points to the critical role of apoptosis in its pathogenic progression. Thus, inhibition of apoptosis can ameliorate the damage caused by I/Rand promote a better functional outcome [39, 40]. In this regards, it has been shown that APOE and its mimetic peptide analogs can reduce neuronal apoptosis in acute brain injury models such as subarachnoid hemorrhage and TBI [14, 37]. The present study tested the possibility that bexarotene can inhibit neuronal apoptosis after cerebral I/R injury. For this purpose, the level of cleaved caspase-3, acritical executor of cell apoptosis was measured in the proximity of the injury site [41]. This analysis indicated that bexarotene treatment significantly reduced the number of TUNEL-positive cells at 3 days after t-MCAO in wild-type C57/BL6 mice, but failed to produce a statistically significant change in APOE-KO mice. On this basis, the possibility was raised that the reduction of apoptosis by bexarotene may be dependent on APOE.

In conclusion, the present study shows that bexarotene alleviates the neurological deficits and improves the histological outcomes associated with transient focal cerebral ischemia in mice. The neuroprotective effects may represent the consequence of inhibition of neuronal apoptosis by suppressing the JNK/caspase-3 signaling pathway. Moreover, the beneficial impact of bexarotene may be due, at least in part, to its ability to up-regulate the expression of APOE. Therefore, bexarotene may be considered as a potential therapeutic agent for the treatment of cerebral ischemia patients.

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Author Contributions LX made a substantial contribution to the conception, design, and execution of the study. HL, JC, XH, RJ, QL, ZF, SW, QZ, YL, WD, and ZD participated in performing the experiments and carried out data analysis. HL participated in the performance of the study and writing the manuscript. All authors have read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Reference

1. Flynn RW, MacWalter RS, Doney AS (2008) The cost of cerebral ischaemia. Neuropharmacology 55:250–256
2. Fonarow GC, Reeves MJ, Zhao X, Olson DM, Smith EE, Saver JL, Schwamm LH, Get With the Guidelines-Stroke Steering Committee and Investigators (2010) Age-related differences in characteristics, performance measures, treatment trends, and outcomes in patients with ischemic stroke. Circulation 121:879–891
3. Huuskonen MT, Loppi S, Dhungana H, Keksa-Goldsteine V, Lemarchant S, Korhonen P, Wojciechowski S, Pollari E, Valonen P, Koponen J, Takashima A, Landreth G, Goldsteins G, Malm T, Koistinaho J, Kanninen KM (2016) Bexarotene targets autophagy and is protective against thromboembolic stroke in aged mice with tauopathy. Sci Rep 6:33176
4. Xu L, Cao F, Xu F, He B, Dong Z (2015) Bexarotene reduces blood-brain barrier permeability in cerebral ischemia-reperfusion injured rats. PLoS ONE 10:e0122744
5. Lynch JR, Morgan D, Mance J, Matthew WD, Laskowitz DT (2001) Apolipoprotein E modulates glial activation and the endogenous central nervous system inflammatory response. J Neuroimmunol 114:107–113
6. Lomnitski L, Chapman S, Hochman A, Kohen R, Shohami E, Chen Y, Trembovler V, Michaelson DM (1999) Antioxidant mechanisms in apolipoprotein E deficient mice prior to and following closed head injury. Biochim Biophys Acta 1453:359–368
7. Zhou S, Wu H, Zeng C, Xiong X, Tang S, Tang Z, Sun X (2013) Apolipoprotein E protects astrocytes from hypoxia and glutamate-induced apoptosis. FEBS Lett 587:254–258
8. Osada N, Kosuge Y, Kihara T, Ishige K, Ito Y (2009) Apolipoprotein E-deficient mice are more vulnerable to ER stress after transient forebrain ischemia. Neurochem Int 54:403–409
9. Cramer PE, Cirrito JR, Wesson DW, Lee CY, Karlo JC, Zinn AE, Casali BT, Restivo JL, Goebel WD, James MJ, Brunden KR, Wilson DA, Landreth GE (2012) ApoE-directed therapeutics rapidly clear beta-amyloid and reverse deficits in AD mouse models. Science 335:1503–1506
10. McFarland K, Spalding TA, Hubbard D, Ma JN, Olsson R, Burstein ES (2013) Low dose bexarotene treatment rescues dopamine neurons and restores behavioral function in models of Parkinson's disease. ACS Chem Neurosci 4:1430–1438
11. Riancho J, Ruiz-Soto M, Berciano MT, Berciano J, Lafarga M (2015) Neuroprotective effect of bexarotene in the SOD1(G93A) mouse model of amyotrophic lateral sclerosis. Front Cell Neurosci 9:250
12. Tunctan B, Kucukkavruk SP, Temiz-Resitoglu M, Guden DS, Sari AN, Sahan-Firat S (2018) Bexarotene, a selective RXRalpha

agonist, reverses hypotension associated with inflammation and tissue injury in a rat model of septic shock. Inflammation 41:337–355
13. Certo M, Endo Y, Ohta K, Sakurada S, Bagetta G, Amantea D (2015) Activation of RXR/PPARgamma underlies neuroprotection by bexarotene in ischemic stroke. Pharmacol Res 102:298–307
14. Zhong J, Cheng C, Liu H, Huang Z, Wu Y, Teng Z, He J, Zhang H, Wu J, Cao F, Jiang L, Sun X (2017) Bexarotene protects against traumatic brain injury in mice partially through apolipoprotein E. Neuroscience 343:434–448
15. Sugo N, Hurn PD, Morahan MB, Hattori K, Traystman RJ, DeVries AC (2002) Social stress exacerbates focal cerebral ischemia in mice. Stroke 33:1660–1664
16. Bachmeier C, Beaulieu-Abdelahad D, Crawford F, Mullan M, Paris D (2013) Stimulation of the retinoid X receptor facilitates beta-amyloid clearance across the blood–brain barrier. J Mol Neurosci 49:270–276
17. Yang Y, Liu P, Chen L, Liu Z, Zhang H, Wang J, Sun X, Zhong W, Wang N, Tian K, Zhao J (2013) Therapeutic effect of Ginkgo biloba polysaccharide in rats with focal cerebral ischemia/reperfusion (I/R) injury. Carbohydr Polym 98:1383–1388
18. Sapkota A, Gaire BP, Cho KS, Jeon SJ, Kwon OW, Jang DS, Kim SY, Ryu JH, Choi JW (2017) Eupatilin exerts neuroprotective effects in mice with transient focal cerebral ischemia by reducing microglial activation. PLoS One 12:e0171479
19. Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, Sanchez-Ramos J, Chopp M (2001) Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. Stroke 32:2682–2688
20. Hamm RJ, Pike BR, O'Dell DM, Lyeth BG, Jenkins LW (1994) The rotarod test: an evaluation of its effectiveness in assessing motor deficits following traumatic brain injury. J Neurotrauma 11:187–196
21. He J, Liu H, Zhong J, Guo Z, Wu J, Zhang H, Huang Z, Jiang L, Li H, Zhang Z, Liu L, Wu Y, Qi L, Sun X, Cheng C (2018) Bexarotene protects against neurotoxicity partially through a PPARgamma-dependent mechanism in mice following traumatic brain injury. Neurobiol Dis 117:114–124
22. Luo Y, Kuang S, Li H, Ran D, Yang J (2017) cAMP/PKA-CREB-BDNF signaling pathway in hippocampus mediates cyclooxygenase 2-induced learning/memory deficits of rats subjected to chronic unpredictable mild stress. Oncotarget 8:35558–35572
23. Aronowski J, Strong R, Grotta JC (1997) Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats. J Cereb Blood Flow Metab 17:1048–1056
24. Bomben V, Holth J, Reed J, Cramer P, Landreth G, Noebels J (2014) Bexarotene reduces network excitability in models of Alzheimer's disease and epilepsy. Neurobiol Aging 35:2091–2095
25. Rosell A, Agin V, Rahman M, Morancho A, Ali C, Koistinaho J, Wang X, Vivien D, Schwaninger M, Montaner J (2013) Distal occlusion of the middle cerebral artery in mice: are we ready to assess long-term functional outcome? Transl Stroke Res 4:297–307
26. Pineiro R, Pendlebury ST, Smith S, Flitney D, Blamire AM, Styles P, Matthews PM (2000) Relating MRI changes to motor deficit after ischemic stroke by segmentation of functional motor pathways. Stroke 31:672–679
27. Thirugnanachandran T, Ma H, Singhal S, Slater LA, Davis SM, Donnan GA, Phan T (2018) Refining the ischemic penumbra with topography. Int J Stroke 13:277–284
28. Petralia RS, Mattson MP, Yao PJ (2014) Communication breakdown: the impact of ageing on synapse structure. Ageing Res Rev 14:31–42
29. Jin R, Liu L, Zhang S, Nanda A, Li G (2013) Role of inflammation and its mediators in acute ischemic stroke. J Cardiovasc Transl Res 6:834–851
30. Tachibana M, Shinohara M, Yamazaki Y, Liu CC, Rogers J, Bu G, Kanekiyo T (2016) Rescuing effects of RXR agonist bexarotene on aging-related synapse loss depend on neuronal LRP1. Exp Neurol 277:1–9
31. Boehm-Cagan A, Michaelson DM (2014) Reversal of apoE4-driven brain pathology and behavioral deficits by bexarotene. J Neurosci 34:7293–7301
32. Fuentes D, Fernandez N, Garcia Y, Garcia T, Morales AR, Menendez R (2018) Age-related changes in the behavior of apolipoprotein E knockout mice. Behav Sci (Basel). https://doi.org/10.3390/bs8030033
33. Chen Y, Lomnitski L, Michaelson DM, Shohami E (1997) Motor and cognitive deficits in apolipoprotein E-deficient mice after closed head injury. Neuroscience 80:1255–1262
34. Bell RD, Winkler EA, Singh I, Sagare AP, Deane R, Wu Z, Holtzman DM, Betsholtz C, Armulik A, Sallstrom J, Berk BC, Zlokovic BV (2012) Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. Nature 485:512–516
35. Mehan S, Meena H, Sharma D, Sankhla R (2011) JNK: a stress-activated protein kinase therapeutic strategies and involvement in Alzheimer's and various neurodegenerative abnormalities. J Mol Neurosci 43:376–390
36. Yang L, Liu CC, Zheng H, Kanekiyo T, Atagi Y, Jia L, Wang D, N'Songo A, Can D, Xu H, Chen XF, Bu G (2016) LRP1 modulates the microglial immune response via regulation of JNK and NF-kappaB signaling pathways. J Neuroinflammation 13:304
37. Wu Y, Pang J, Peng J, Cao F, Vitek MP, Li F, Jiang Y, Sun X (2016) An apoE-derived mimic peptide, COG1410, alleviates early brain injury via reducing apoptosis and neuroinflammation in a mouse model of subarachnoid hemorrhage. Neurosci Lett 627:92–99
38. Hoe HS, Harris DC, Rebeck GW (2005) Multiple pathways of apolipoprotein E signaling in primary neurons. J Neurochem 93:145–155
39. Liu B, Li F, Shi J, Yang D, Deng Y, Gong Q (2016) Gastrodin ameliorates subacute phase cerebral ischemiareperfusion injury by inhibiting inflammation and apoptosis in rats. Mol Med Rep 14:4144–4152
40. Liu G, Wang T, Wang T, Song J, Zhou Z (2013) Effects of apoptosis-related proteins caspase-3, Bax and Bcl-2 on cerebral ischemia rats. Biomed Rep 1:861–867
41. Na KS, Park BC, Jang M, Cho S, Lee DH, Kang S, Lee CK, Bae KH, Park SG (2007) Protein disulfide isomerase is cleaved by caspase-3 and -7 during apoptosis. Mol Cells 24:261–267