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ORIGINAL ARTICLE

DAPT protects brain against cerebral ischemia by down-regulating the expression of Notch 1 and Nuclear factor kappa B in rats

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Abstract Gamma-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-1-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) suppresses the activation of Notch 1 signaling, which is recognized as the cell fate signaling and may participate in inflammatory processes together with NF-κB pathway that contributes to the brain damage after stroke. DAPT has important pharmacological roles in many diseases. However, little is known about the effect of DAPT on NF-κB during cerebral ischemia. This study investigated the time course expression of Notch 1 and the effects of DAPT on Notch 1 and NF-κB after MCAO. The results showed that Notch 1 signaling was up-regulated at the early stage after MCAO, DAPT down-regulated the expression of Notch 1 and NF-κB and protected brain from damage caused by MCAO. These results may indicate that the downregulation of Notch 1–NF-κB pathway after ischemia by administration of DAPT is a potential mechanism for its protection.

Keywords Cerebral ischemia · DAPT · Notch 1 · Nuclear factor kappa B · Inflammation

Introduction

The Notch 1 signaling pathway is of fundamental importance in a wide variety of pathophysiological processes in adults including apoptosis and inflammatory processes, which are parts of the mechanism of brain injury resulting from cerebral ischemia. One of γ-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-1-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), efficiently inhibiting the activation of the Notch 1 signaling [1–5], has been used to treat neurodegenerative diseases [3, 6, 7] and modulated the differentiation of neural progenitor and the apoptotic cascades in neurons in cerebral ischemia which contribute to the neuroprotection of DAPT [8, 9]. Little is known weather DAPT protects brain from cerebral ischemia by interfering in inflammatory processes. NF-κB, a family of transcription factors [10, 11], participated in ischemic injury by promoting inflammatory processes and inducing the apoptosis of neurons [12]. But study on the relationship between Notch1 and NF-κB is sparse [13, 14]. The present project is to examine whether DAPT administration can protect cerebral ischemia and whether Notch1 and NF-κB are involved in DAPT protection in cerebral ischemia in rat.

Materials and methods

Animals

Male Sprague–Dawley rats (260–290 g) were purchased from Hebei Medical University. The protocol was approved by the institutional animal care and use committee and the local experimental ethics committee. All rats were allowed free access to food and water under controlled conditions (12/12 h light/dark cycle with humidity of 60 ± 5%, 22 ± 3°C).

Rat model of permanent focal cerebral ischemia

A modified model of MCAO was used to make permanent focal ischemia as previously described [15, 16]. Animals

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were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Body temperature was monitored and maintained at 36.5–37.5°C. Briefly, after midline skin incision, the right common carotid artery (CCA) and external carotid artery (ECA) were exposed and isolated by blunt dissection. ECAs were dissected and the distal branches ligated. The right middle cerebral artery (MCA) was permanent occluded by intraluminal placement of filament, as described previously. The common right carotid artery was exposed and isolated. MCA was occluded by inserting a filament into the internal carotid artery, which was advanced further until it closes the origin of the MCA. Sham-operated control rats received the same procedure except for intraluminal insertion of the filament.

Groups and drug administration

Experiment 1: dynamic expression of Notch 1 in cerebral ischemia

42 rats were randomly assigned to seven time course groups ($n = 6$ in each group), including a normal-control group (Normal) and 3, 6, 12, 24, 48 and 72 h after MCAO. Immunohistochemistry and Western blot were used to analyze the dynamic expression of Notch 1.

Experiment 2: DAPT's protective effect in the acute phase of cerebral ischemia

DAPT powder (Sigma-Aldrich, St. Louis, MO, USA), was dissolved in 0.01 M phosphate-buffered saline (PBS) including 5% DMSO to prepare concentrations of 8.3 mg/ml. DAPT solution was stereotactically injected into the lateral cerebral ventricle (LV) immediately after MCAO. The stereotactic injections into the LVs were performed at coordinates −0.8 mm anteroposterior, ±1.5 mm mediolateral and −4.5 mm dorsoventral from the bregma. 30 rats were randomly assigned to three operating groups (10 rats in each group): sham-operated group that received equal volume of PBS without MCAO operation (Sham); MCAO group that received equal volume PBS after MCAO (MCAO); and DAPT group that received DAPT as 0.03 mg/kg after MCAO. 24 h after operation the first neurological function was assessed and then 48 h after operation the second neurological function was assessed. Meanwhile, brain water content and infarction volume were measured and compared among different groups.

Experiment 3: the effect of DAPT on Notch 1 and NF-κB

In this part rats were still randomly divided into three groups as Sham group, MCAO group and DAPT group ($n = 9$ for each group). Rats were reanesthetized and killed 48 h after being successfully operated. Immunohistochemistry, Western blot and Immunofluorescence were used to detect the expression of Notch 1 and NF-κB.

Analysis of neurological deficit scores

A neurological test was carried out by an examiner blinded to the experimental groups. The deficits were scored on a modified scoring system based on that developed by Longa et al. (1989) as follows: 0, no deficits; 1, difficulty in fully extending the contralateral forelimb; 2, unable to extend the contralateral forelimb; 3, mild circling to the contralateral side; 4, severe circling; and 5, falling to the contralateral side. Rats with 0, 1 and 5 were excluded from the study.

Measurement of brain water content

Following neurological behavior tests, rats were killed at 3, 6, 12, 24, 48 and 72 h after operation. Brain tissues were rapidly obtained and first weighed wet on an electronic balance and then dried for 24 h at (100 ± 5)°C to measure dry weight. Results were calculated as follows: brain water content = (wet weight − dry weight)/wet weight × 100% [17].

Measurement of infarction volume

Rats were re-anesthetized after neurological behavior test at each time point and the brains were removed quickly. Coronal brain sections (2 mm thick) were stained with 2% TTC at 37°C for 20 min. The stained cerebral sections were photographed and ipsilateral and contralateral hemispheric volumes and infarct volumes were quantified with the use of Image Pro-Plus 5.1 analysis system. To compensate for the effect of brain edema, the infarction volumes were calculated by the following equations. Percentage hemisphere lesion volume (%HLV) = {[total infarct volume − (the volume of intact ipsilateral hemisphere − the volume of intact contralateral hemisphere)]/contralateral hemisphere volume} × 100% [18].

Immunohistochemistry

Brains were removed quickly, immersed with 4% paraformaldehyde in 0.01 M PBS for 3–7 days at ordinary temperature. Following embedding in paraffin, the tissues were serially sectioned in 5-μm-thick slices for application to the standard histological procedure as follows: the slices were blocked in 3% H_2O_2, 3% normal goat serum and incubated with Notch 1 rabbit polyclonal antibody (1:400, Abcam Biotechnology), NF-κB P65 rabbit polyclonal antibody (1:150, Santa Cruz Biotechnology) in 0.01 M phosphate-buffered saline over night. The secondary antibodies, secondary biotinylated conjugates and diaminobenzidine were

from the Vect ABC kit (Zhongshan Biology Technology Company, China). Five visual fields of ischemic region of the infarct were selected and the immunoreactive cells were counted under a 400× light microscope.

Western blot

Rats were killed by decapitation under anesthesia. Total protein was extracted from infarction zone and peri-infarction cortex using a Total Protein Extraction Kit (Applygen Technologies Inc., Beijing) following the manufacturer's protocols. Then the protein concentration was determined using a BCA Protein Assay reagent kit (Novagen, Madison, WI, USA). For polyacrylamide gel electrophoresis (PAGE), samples were boiled at 100°C for 5 min after the addition of the sample buffer. 50 μg of proteins was separated by SDS/PAGE, transferred 2 h on to PVDF membranes and the nonspecific binding of antibodies was blocked with 5% non-fat dry milk in PBS. Membranes were then probed with Notch 1 rabbit polyclonal antibody (1:300, Abcam Biotechnology), NF-κB P65 rabbit polyclonal antibody (1:100, Santa Cruz Biotechnology) overnight at 4°C. Membranes loaded with primary antibodies were washed with 0.1% tween-20 Tris-buffered saline and then were incubated with fluorescent labeling second antibodies (goat anti-rabbit, 1:8,000, Rockland, Gilbertsville, PA, USA) for 1 h at room temperature. An imaging densitometer (LI-COR Bioscience) was used to analyze the relative density of each band. Anti-rat β-actin (1:500, Zhongshan Biotechnology) was used as internal control.

Immunofluorescence

First, nylon monofilaments inserted into ICA were pulled out, and brains were perfused transcardially with saline quickly followed by 4% paraformaldehyde. Frozen coronal sections (30-μm-thick) were prepared at −20°C. After soaking in 0.3% Triton X-100 for 10 min and blocking with 10% normal horse serum for half an hour, brain sections were incubated with Notch 1 rabbit polyclonal antibody (1:300, Abcam Biotechnology), NF-κB P65 rabbit polyclonal antibody (1:100, Santa Cruz Biotechnology) at 4°C overnight. On the second day, slices were incubated with secondary antibody (anti-rabbit FITC, 1:100 dilution, Beijing) and nuclear marker Hoechst 33342 for 1 h and then were observed under 20× Laser Scanning Confocal Microscope (Olympus FV10-ASW, Japan).

Statistical analysis

All data were analyzed using SPSS 13.0 software. Results were expressed as mean ± SD deviation using one-way analysis of variance. Data were analyzed with ANOVA and followed by SNK and LSD tests for intergroup comparisons. Mann–Whitney U test was used for comparisons of neurological deficit between two groups. Differences were considered significant if $P < 0.05$.

Results

Notch 1 was up-regulated in the acute phase of cerebral ischemia

Immunohistochemistry and Western blot were used to detect the dynamic expression of Notch 1 in brain tissue at normal, 3, 6, 12, 24, 48 and 72 h after permanent occlusion of the middle cerebral artery (MCAO) (Figs. 1, 2). Compared with normal-control group, Notch 1 was up-regulated beginning at 3 h ($P < 0.05$), getting to high values at 24 h, peaking at 48 h and maintaining high values at 72 h after MCAO ($P < 0.05$) in protein levels. All the results of immunohistochemistry and Western blot showed that, compared with 3, 6 and 12 h after permanently MCAO, the expression of Notch 1 at 24 h was significantly increased ($P < 0.05$), but slightly lower than peak values.

The effect of DAPT on neurological deficit scores

Neurological deficit was examined and scored on a 6-point scale at 24 and 48 h after MCAO, and then Mann–Whitney U test was conducted. Although there were significant differences in neurological deficit scores between DAPT group and MCAO group at 48 h ($P < 0.05$, Fig. 3b), there was no significant reduction at 24 h ($P > 0.05$, Fig. 3a).

DAPT reduced the brain water content

We observed brain water content at 48 h after the operation using the standard wet–dry method [17]. DAPT could reduce the brain water content of ipsilateral hemispheres. In the sham-operated group, water content was 78.83 ± 0.35%. In DAPT group, brain water content was reduced compared with MCAO group (80.89 ± 0.51 vs. 83.84 ± 0.75%, $P < 0.05$). These data indicated that DAPT protected brain against brain ischemia damage at the early stage of cerebral ischemia (Fig. 4a).

DAPT reduced the infarct volume

The protective effects of DAPT were also evaluated by measuring infarct volumes at 48 h after ischemia. We examined infarct sizes at 48 h after operation using vital

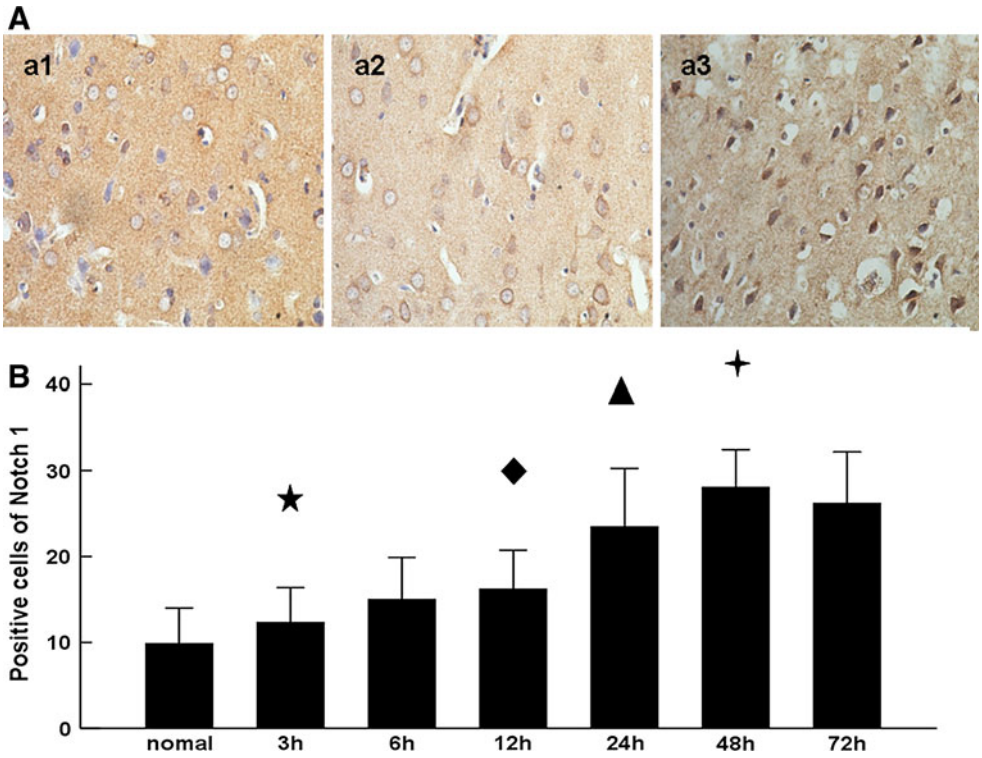

Fig. 1 The dynamic expression of Notch 1 after MCAO. **a** Representative photographs of immunohistochemistry for Notch 1 (400×). a_1 Normal group. a_2 24 h group. a_3 48 h group. **b** *Bar graph* of immunohistochemistry illustrating the dynamic expression of Notch 1. $^{\star}P < 0.05$ versus normal group. $^{\blacklozenge}P < 0.05$ versus normal group, 3 h. $^{\blacktriangle}P < 0.05$ versus normal group, 3, 6, 12 h. $^{*}P < 0.05$ versus normal group, 3, 6, 12, 24, 72 h

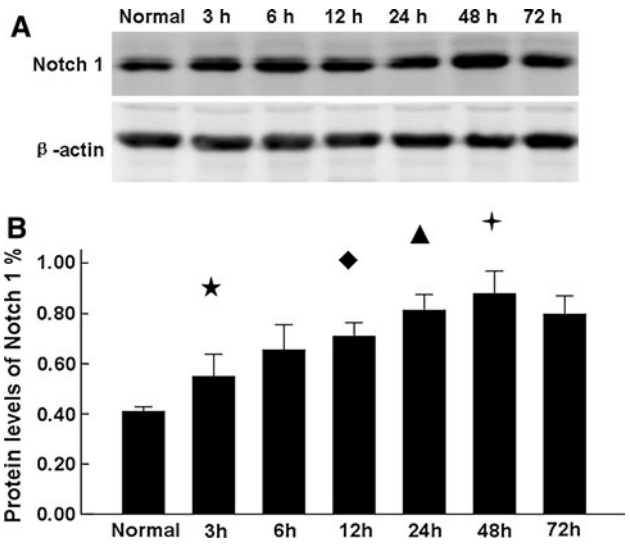

Fig. 2 **a** Representative photographs of Western blot of Notch 1. **b** *Bar graph* of Western blot illustrating the dynamic expression of Notch 1. $^{\star}P < 0.05$ versus normal group. $^{\blacklozenge}P < 0.05$ versus normal group, 3 h. $^{\blacktriangle}P < 0.05$ versus normal group, 3, 6, 12 h. $^{*}P < 0.05$ versus normal group, 3, 6, 12, 24, 72 h

staining with 2, 3, 5-triphenyltetrazolium chloride (TTC). No infarction was observed in the sham-operated group. Extensive lesion was developed in both striatum and lateral cortex in MCAO group. In DAPT group, the infarct volume was decreased significantly from 52.41 ± 1.41 to 41.50 ± 1.93% ($P < 0.05$, Fig. 4b, c).

DAPT suppressed the expression of Notch 1 and NF-κB

The expression of positive cells of Notch 1 and NF-κB were observed in ischemic cortex around infarct regions at 48 h post-ischemia with treatment of DAPT. Immunohistochemistry showed that the number of positive cells of Notch 1 and NF-κB dramatically increased in ischemic cortex in MCAO group, while the DAPT group showed a much lower number ($P < 0.05$, Fig. 5). Western blot analyses also showed a significant decrease of Notch 1 and NF-κB expression in DAPT group ($P < 0.05$ vs. MCAO group Fig. 6), which was consistent with the result of immunohistochemistry. We next examined the expression of Notch 1 and NF-κB at 48 h after MCAO with or without DAPT using confocal microscope in order to confirm the inhibition of Notch 1 by DAPT. Immunofluorescent intensity showed a significant increase of Notch 1 and NF-κB immunoreactivity after MCAO, while the DAPT group again showed a lower intensity (Fig. 7).

Discussion

Our study showed that Notch 1 was up-regulated at early stage after ischemia, beginning at 3 h, peaking at 48 h and maintaining high levels till 72 h when we ceased to observe. These results indicated that Notch 1 pathway participated in the pathologic process of cerebral ischemia at early time. Notch 1 is a double-edged sword in cerebral

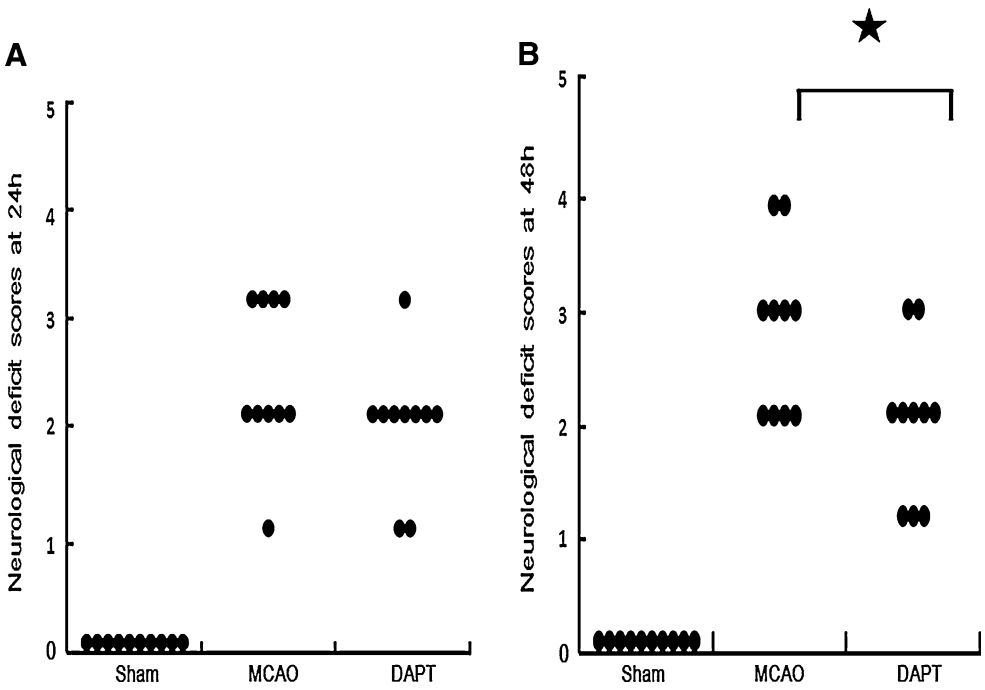

Fig. 3 Effects of DAPT on neurological deficit scores after MCAO. $^{\star}P < 0.05$

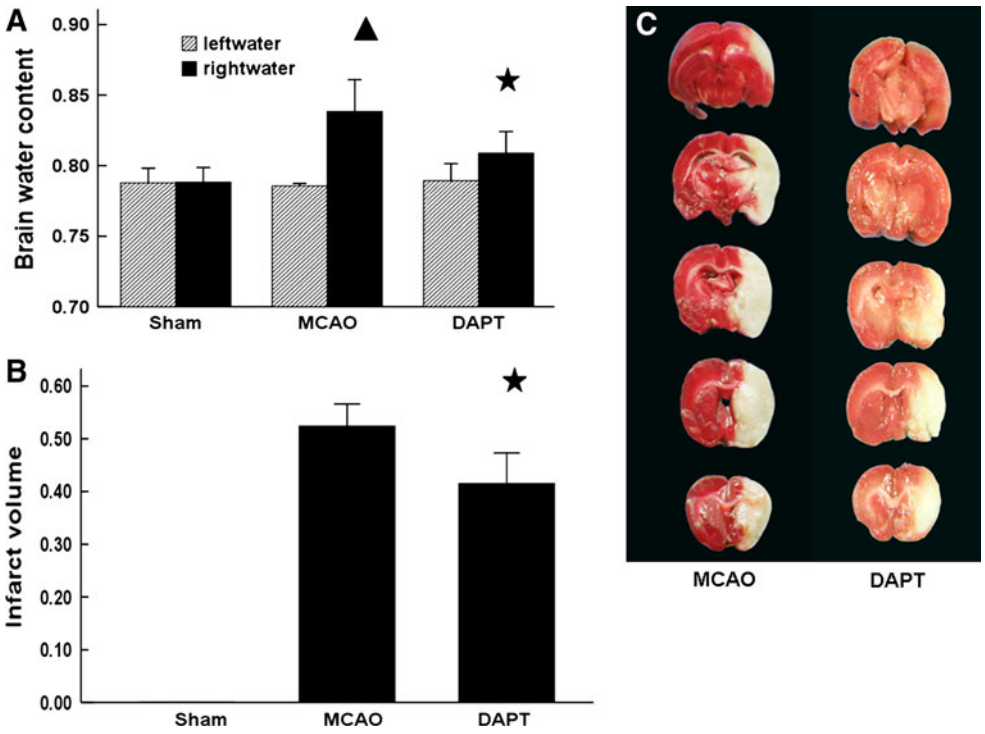

Fig. 4 **a** Effects of DAPT on brain water content. **b** Effects of DAPT on infarct sizes. **c** TTC staining for brain slices. $^{\blacktriangle}P < 0.05$ versus Sham group, $^{\star}P < 0.05$ versus MCAO group

ischemia according to earlier studies. It is reported that Notch 1 stimulated regenerative responses in low-oxygen conditions by maintaining NSCs, promoting proliferation of neural progenitors, but inhibiting differentiation into neurons in vitro and in vivo experiments [19–24]. On the other hand, Notch 1 inhibits neural progenitor differentiation into neurons in vitro and in vivo experiments and participates in various aspects of inflammatory reactions by modulating the development and activation of inflammatory cells, such as T cell, lymphocyte and microglia [25–30]. The balance of Notch 1 pathway may influence the degree of brain injury after cerebral ischemia. DAPT, the inhibitor of Notch 1 pathway, was used in our study to investigate the final function of Notch 1 pathway in cerebral ischemia. Neurological deficit, brain water content and infarct sizes were measured 48 h after MCAO. Data

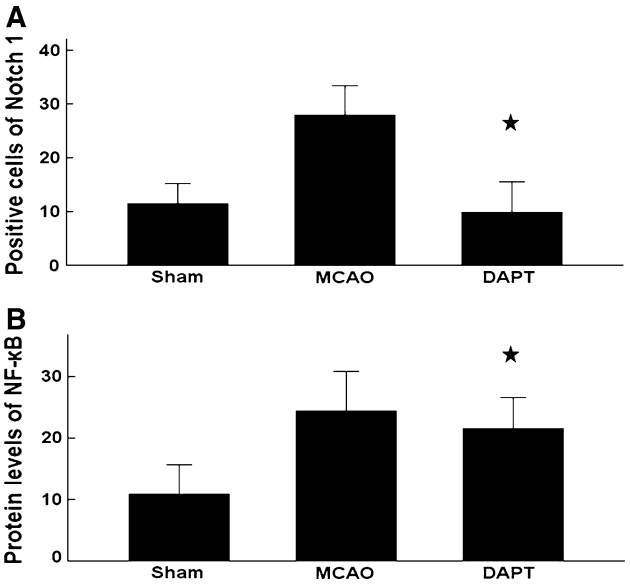

Fig. 5 **a** *Bar graph* showing the effect of DAPT on number of positive cells of Notch 1. **b** *Bar graph* showing the effect of DAPT on number of positive cells of NF-κB. $^{\star}P < 0.05$ versus MCAO group

showed that DAPT reduced brain water content and infarct volumes and improved the functional outcome after cerebral ischemia. Notch 1 might be benefit for neural regeneration; however, DAPT inhibiting Notch 1 protected brain damage from ischemic stroke. It implicated that Notch 1 participating in inflammatory processes may play a major role in cerebral ischemia or DAPT may regulate other signaling pathway.

Activated NF-κB is transferred to the nucleus, where it combines with target genes to promote proinflammatory cytokine expression which results in leukocyte adherence and migration, as well as expanded inflammatory reaction. NF-κB is a key factor contributing to the brain damage after ischemic stroke [12]. Numerous reports have described that Notch 1 regulated NF-κB and vice versa. NF-κB could modulate and integrate into Notch 1 pathway through Notch ligands Jagged-1 and intracellular Notch modulators N-CoR. Notch 1 regulated NF-κB via Jagged-1 and NF-κB modulators IκBα [31–33]. Several lines of evidences supported a consonance of the Notch and NF-κB signaling pathways in activation and function [13, 14], and both of them are positively activated by ischemia. Our observations showed that DAPT significantly inhibited the expression of NF-κB at protein level. Other researchers provided parallel results about γ-secretase inhibitors [34].

Previous studies have demonstrated that DAPT may present an effective and novel treatment for autoimmune and lymphoproliferative diseases, degenerative disease and cancers [27, 35–37]. The neuroprotection of DAPT in cerebral ischemia by decreasing the apoptotic cascades in neurons, restraining the activation of microglias and repressing the infiltration of proinflammatory leukocytes has been demonstrated [30].

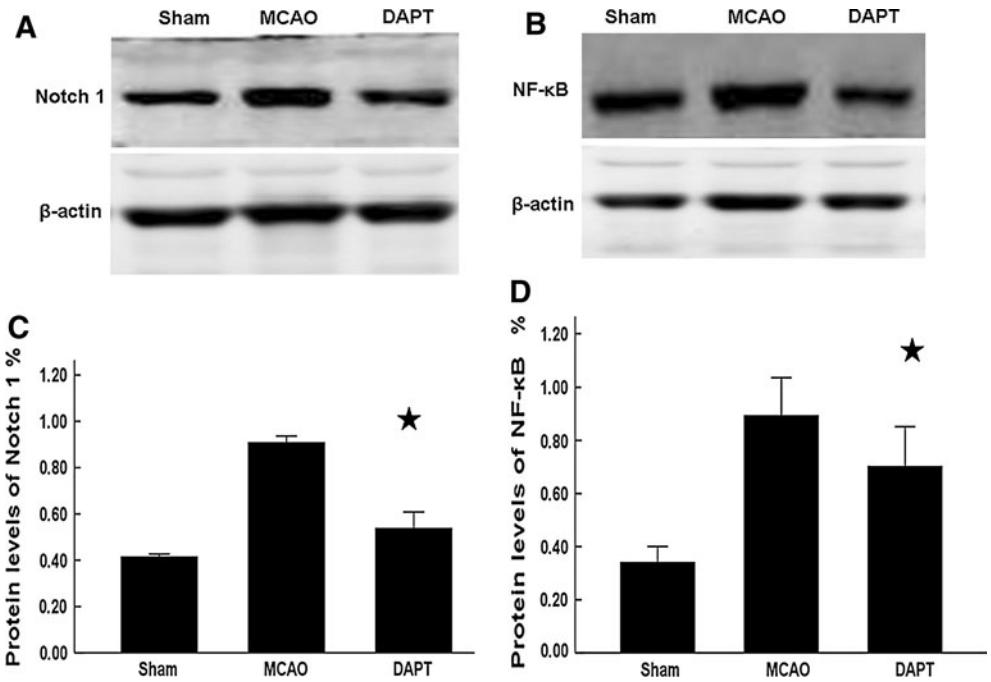

Fig. 6 **a** Representative photographs of Western blot showing the effect of DAPT on Notch 1 protein levels. **b** Representative photographs of Western blot showing the effect of DAPT on NF-κB protein levels. **c** *Bar graph* showing the effect of DAPT on Notch 1 protein levels. **d** *Bar graph* showing the effect of DAPT on NF-κB protein levels. $^{\star}P < 0.05$ versus MCAO group

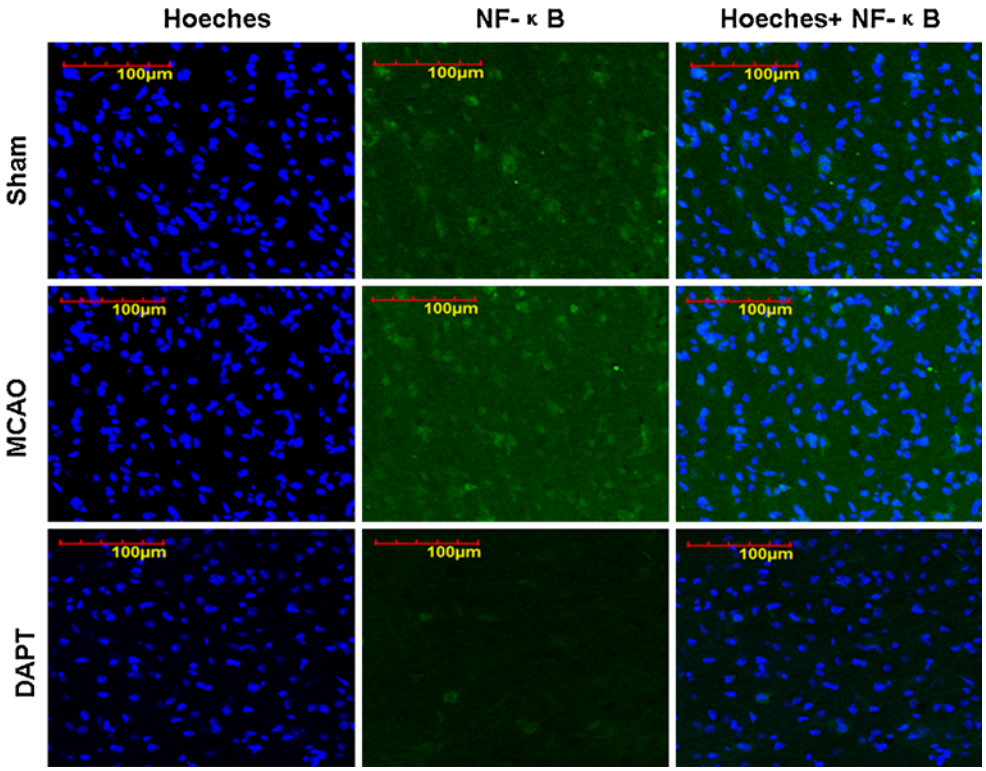

Fig. 7 Confocal microscope showed that DAPT decreased the expression of NF-κB

In summary, the results showed that the regularity of the time course expression of Notch 1 upregulated at very early date after cerebral ischemia. Systemic administration of DAPT decreased the infarct size and the brain edema. These results may indicate that the downregulation of Notch 1–NF-κB pathway after ischemia by administration of DAPT is a potential mechanism for its protection.

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