Nimodipine improves spatial memory via inhibiting NLRP3 inflammasome activation in a rat model of focal cerebral ischemia

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Abstract: Impairment of spatial memory is a common complication after cerebral ischemia. This study was performed to evaluate the neuroprotective effect of nimodipine on spatial memory in a rat model of focal cerebral ischemia. A rat model of focal cerebral ischemia was induced in Sprague-Dawley male rats by middle cerebral artery occlusion (MCAO). The model rats were treated with intracarotid infusion of 40 μg/kg/30 min nimodipine (started 15 min before recirculation and maintained until 15 min after recirculation) plus intraperitoneal injection of 2 mg/kg nimodipine daily in the next three weeks. The negative control rats and sham rats received only saline. The spatial memory was then assessed by the Morris water maze. Histological changes of brain were assessed by TUNEL staining and Golgi staining. The NLRP3 inflammasome activation status was assessed by Western blot. Water maze test showed that spatial learning and memory abilities were impaired by MCAO. Nimodipine treatment could reduce escape latency in the spatial acquisition trail and increase cross duration in probe quadrant. Moreover, nimodipine attenuated neuron cell apoptosis in the hippocampus area. Golgi-staining showed that nimodipine also improved neuron morphology in the hippocampus and increased dentritic spine density in the CA1 area. In addition, nimodipine reduced expression of NLRP3,

cleaved caspase-1, IL-1β, and IL-18, indicating that NLRP3 inflammasome activation was attenuated. In conclusion, nimodipine has neuroprotective effect in rat model of focal cerebral ischemia by inhibiting NLRP3 inflammasome activation.

Keywords: focal cerebral ischemia, NLRP3 inflammasome, Morris water maze, hippocampus, nimodipine

Introduction

Cerebrovascular disease is a common chronic disease in clinic. The morbidity and mortality of cerebrovascular disease have exceeded other diseases, making cerebrovascular a big threat of human health (1). Cerebral ischemia is the most common cerebrovascular disease, accounting for about 85% of all cases. The main manifestation of cerebral ischemia is the insufficient blood circulation and the following onset of a persistent neurologic deficit. The pathological process is different between the central area and the surrounding area of cerebral ischemia. Irreversible cell necrosis is often found in the nerve cells of the central area due to severe ischemia and hypoxia, but reversible apoptosis occurs more frequently in the ischemic penumbra at the edge of the infarct. Inhibition of neuron apoptosis in the ischemic penumbra, restoration of neural function, and reduction of the extent of brain tissue damage are the main strategies for the treatment of cerebral ischemic (2, 3).

Nimodipine (N-methyl-d-aspartate, NMDA), a Ca^{2+} antagonist, has been used to treat cerebral ischemia for decades. Many preclinical and clinical studies have shown that nimodipine directly protects against brain neuronal cell damage by inhibiting excessive Ca^{2+} influx into the mitochondria and controlling the intracellular Ca^{2+} cascade (4-6). In addition, nimodipine is also a treatment for vascular dementia, which can attenuate depression, improve consciousness and memory function (7-9). Post-stroke cognitive impairment occurs frequently and affects 20% to 80% of the stroke patients depending on the demographic factors and diagnostic criteria (10). A phase IV multicenter double-blind, placebo-controlled, randomized trial evaluated the safety and efficacy of nimodipine in preventing cognitive impairment in ischemic stroke patients (11). Although nimodipine (90 mg/d) did not show benefit to prevent cognitive decline in acute ischemic stroke patients with mild vascular mild cognitive impairment, but did improve cognition moderately, especially in the memory domain (12). These results imply that nimodipine can improve memory function after ischemic stroke, but the molecular mechanism remains unclear.

Recent studies have suggested that NLRP3 (nucleotide-binding domain,

leucine-rich-repeat-containing family, pyrin domain-containing protein 3) inflammasome is activated in Alzheimer's disease and is associated with spatial memory (13, 14). Ca^{2+} mediated mitochondrial damage is a stimulus of NLRP3 inflammasome and blocking Ca^{2+} mobilization inhibits assembly and activation of NLRP3 inflammasome complex (15, 16). Based on the previous studies, we speculate that nimodipine might be able to improve spatial memory by inhibiting the activation of NLRP3 inflammasome, and we investigated this problem in a rat model of focal cerebral ischemia.

Materials and methods

Animals and chemicals

Spraque-Dawley male rats weighing 250-320 g were used in this study. Rats were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). Rats were housed with free access to standard rodent diet and clean water at a controlled circumstance condition (22±2℃, 12:12 h light/dark cycle). The experiment protocol was approved by the Institutional Committee Board of Animal Work. All applicable guidelines for the care and use of laboratory animals were followed. Nimodipine solution was purchased from Beyer (Leverkusen, Germany).

Induction of MACO model and drug administration

Animal model of focal cerebral ischemia was established by middle cerebral artery occlusion (MCAO) (17). Briefly, rats were anesthetized with 10% chloral hydrate (350 mg/kg). The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed via a midline incision and carefully isolated. The pterygopalatine branch of ICA was sealed with electric coagulator. The CCA was ligated distally and the ECA was ligated proximally to the bifurcation of the ICA and the ECA. A heparin-dampened monofilament nylon suture (diameter 0.24-0.26 mm) was inserted into the ICA lumen through the ECA lumen until the tip of the filament blocked the origin of the MCA. The rats' body temperature was maintained at 37℃ during the 90 minutes of ischemia. The filament

was them gently withdrawn to establish reperfusion. The sham rats received the same procedures without inserting a filament. After the rats wake up completely, the neural function was evaluated by Bederson method (18), and the rats with scores≥3 were remained for drug administration. Totally 24 rats were included and randomly divided into nimodipine group and saline group. The nimodipine rats received intracarotid infusion of 40 μg/kg/30 min nimodipine (started 15 min before recirculation and maintained until 15 min after recirculation) plus intraperitoneal injection of 2 mg/kg nimodipine daily in the next three weeks. The drug administration method aims to impregnate the ischemic cerebral tissue just before recirculation through arterial route and to alleviate the deleterious effects caused by ischemia/reperfusion by regular intraperitoneal route (19). The negative control rats and sham rats received only saline.

Spatial memory assessment

After three weeks of treatment, spatial memory of rats was evaluated by the Morris water maze (20, 21). Animals were firstly assessed with 5 days of spatial acquisition trial in a circular pool (60 cm depth × 120 cm diameter) filled with water at 25±2℃. A platform (15-cm diameter) was located 2 cm under the water level. The test is predicated on the rat finding the hidden platform to avoid swimming. The upper limit of the escape latency time was 2 min. On the sixth day, rats received spatial probe trial with the platform retracted. Each rat was allowed to swim for one minute to measure the time spent in the target quadrant and cross times of target quadrant.

Brain tissue preparation

After finishing water maze test, six rats of each group received intracardial perfusion under anesthesia with saline, followed by 4% paraformaldehyde in 10% buffered formalin phosphate for 1h. Brains were removed from the skull and post-fixed in the same fixative overnight at 4℃. Brain samples were dehydrated and embedded in paraffin for TUNEL assay. Three rats of each group were used for Golgi staining. Hippocampus tissues were isolated from cerebral cortex, rinsed with 0.9% saline, and processed with Golgi staining. The hippocampus tissues of the other rats

were removed, quick frozen in liquid nitrogen, and stored at -80℃ for total protein extraction.

Histological assessment (TUNEL and Golgi staining)

For TUNEL assay, brain samples in paraffin were cut into 5-μm sections. After normal deparaffining and rehydration, sections were processed with proteinase K followed by 2% H_2O_2 in methanol. Sections were then processed for detection of DNA fragment and apoptotic bodies with TUNEL staining kit (Roche, Mannheim, Germany) following the instruction. The signal was visualized by streptavidin-biotin-peroxidase complex and diamiobenzidine. Positive cells of 5 different fields were counted under light microscopy (100× magnification).

For Golgi staining, hippocampus tissues were processed by FD Rapid Golgi Stain Kit (FD NeuroThechnologies, Columbia, MD, USA) as instructed. Briefly, fresh hippocampus tissues were immersed in solution A and B in darkness for 2 weeks, then transferred into solution C for 3 days. Hippocampus tissues were sectioned into 100-μm sections at -20℃, dehydrated with ethanol series, and clarifies with xylene. Section slides were observed under light microscopy using an oil immersion lens. CA1 apical dentritic spines were analyzed from 20-μm segments of four distinct branches per neuron and six neurons were analyzed per brain (20).

Western blot

The isolated hippocampus tissue was homogenized in 5-fold volume of protein extraction buffer. The protein concentration was determined by BCA protein assay reagent kit (Novagen, Madison, WI, USA). Equal amount of protein (50 μg) was isolated by10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% BSA and incubated with corresponding primary antibodies: rabbit anti-NLRP3 (1:1000), rabbit anti-caspase 1 (1:500), rabbit anti-1β (1:1000), and rabbit anti-18 (1:1000). Rabbit anti-beta actin (1:2000) was used as internal control. The blots were then probed with HRP-conjugated goat anti-rabbit IgG (1:4000). The protein bands were visualized using an ECL kit (Boster Biological Technology, Wuhan, China). A

density analysis of the bands was performed by ImageJ software.

Statistical analysis

All data were analyzed by GraphPad Prism 5 software and expressed as mean ± SEM (standard deviation). Statistical evaluation of spatial acquisition trial was assessed by two-way ANOVA; other comparisons were performed using one-way ANOVA followed by Tukey's post-hoc. A value of $P<0.05$ was considered to be statistically significant.

Results

Nimodipine treatment improved spatial memory deficit after focal cerebral ischemia

First, we detected the effect of nimodipine on spatial memory in MCAO rats. As shown in Figure 1A, there were significant differences in the ability of spatial acquisition among the three groups. Two-way ANOVA showed significant effect of treatment ($F=18.16$, $P<0.0001$) and training days ($F=12.00$, $P<0.0001$). There was no significant interaction ($F=1.907$, $P=0.0621$). Bonferroni post-hoc test showed significant impaired spatial acquisition ability in the saline group compared with the sham group especially on day 3 and day 4 ($P<0.001$ and $P<0.01$, respectively). Whereas nimodipine effectively improved spatial acquisition and showed no difference of escape latency compared with the sham group. By the last training day, all groups located the platform with the same escape latency. After finishing spatial acquisition test, the platform was removed and the animals further received spatial memory test (Figure 1B-1D). The saline group showed significant impaired memory as less cross times of target quadrant and lower percentage of time spent in target quadrant (both $P<0.01$). The performance of the nimodipine group was not different from the sham group. All the rats spent longer time in the target quadrant than in other quadrants ($F=13.47$, $P<0.0001$), but there was no significant difference in other quadrants, indicating that all the rats had acquired certain spatial memory. The Morris water maze test showed that spatial memory was impaired after focal cerebral

ischemia and the deficit could be restored by nimodipine treatment.

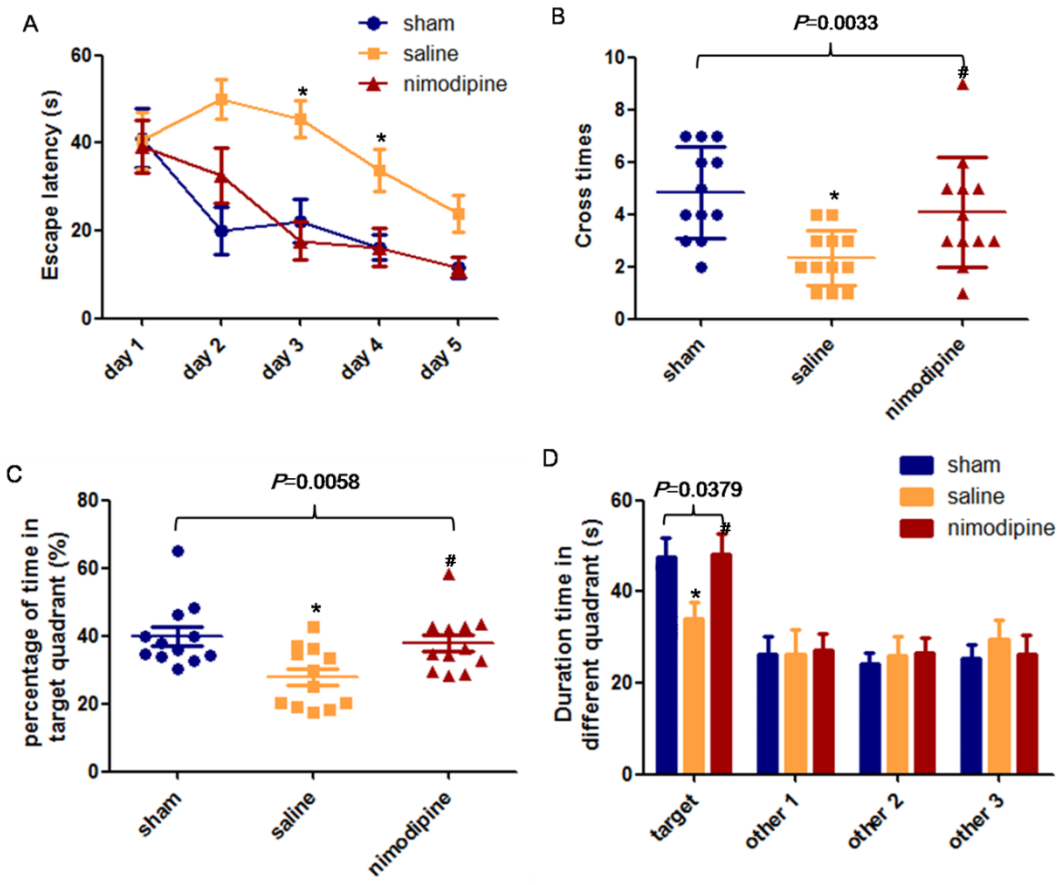

Figure 1. Performance of Morris water maze in different groups

A: Escape latency in spatial acquisition test. B: Cross times through target quadrant in spatial memory test. C: Percentage of duration time spent in target quadrant. D: Duration time spent in all four quadrants. *P<0.05 vs. sham; $^{\#}P$<0.05 vs. saline.

Nimodipine reduces neuronal damage in the hippocampus of MCAO rats

Because hippocampus plays an important role in spatial acquisition/memory, we then investigated the neuron morphology in the hippocampus region. TUNEL staining (Figure 2) showed that more apoptotic cells appeared in the CA1, CA3, and DG regions of the saline group than in those regions of the sham group (all P<0.05). The apoptosis induced by MCAO was reduced by nimodipine treatment. The morphology of apical dentritic spines was further studied by Golgi-staining. The results showed that the number of neuron cells decreased significantly in the saline group than in the sham group, and the number and the length of bifurcation also decreased in the saline

group (Figure 3A). The CA1 region was a crucial area for cognitive ability, so we further quantified the apical spine density and basal branch density in this area (Figure 3B-3C). MCAO induced decrease in CA1 apical and basal spine density by approximately 50% (both $P<0.05$). Nimodipine treatment significantly improved the morphology of hippocampal neuron cells and restored the dentritic spine density in CA1 area to the same level of the sham group.

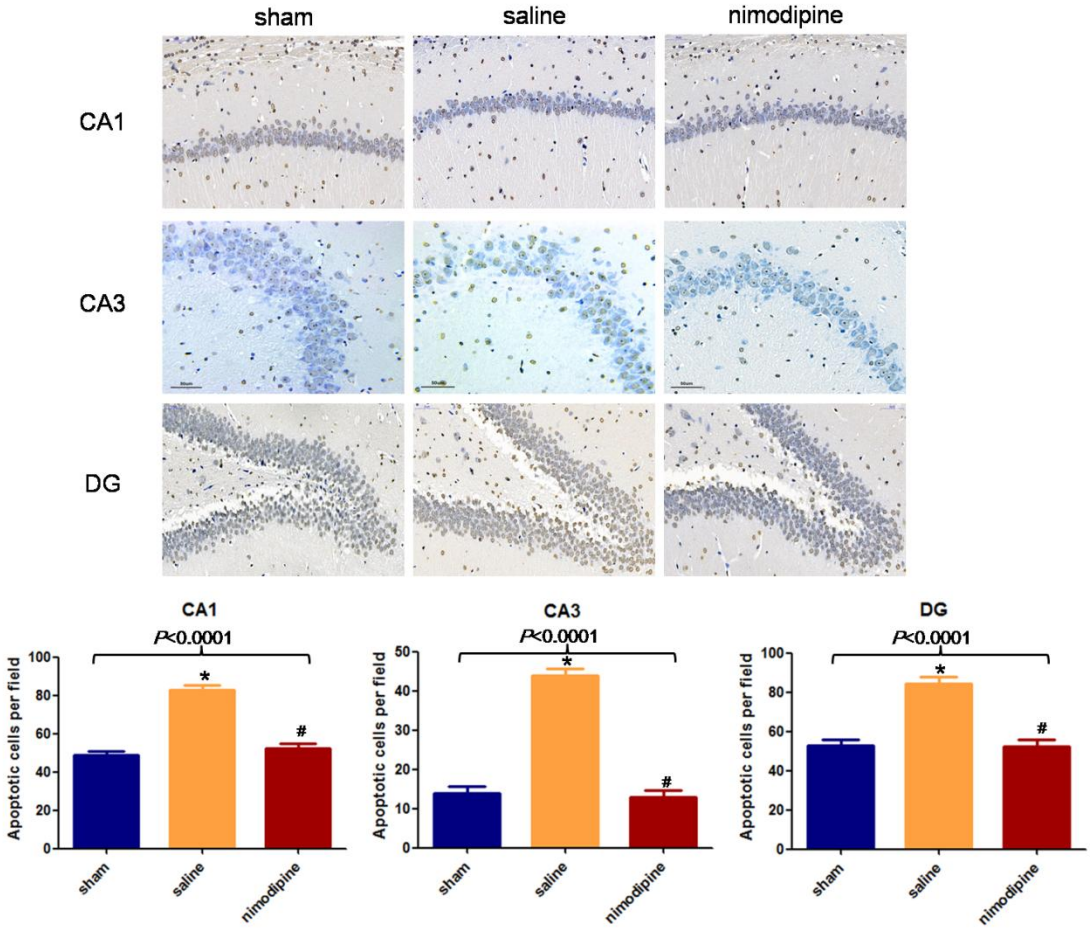

Figure 2. Detection of apoptotic cells in hippocampus by TUNEL assay

Typical sections of CA1, CA3, and DG regions were shown. Scale bar = 50 μm. *$P<0.05$ vs. sham; #$P<0.05$ vs. saline.

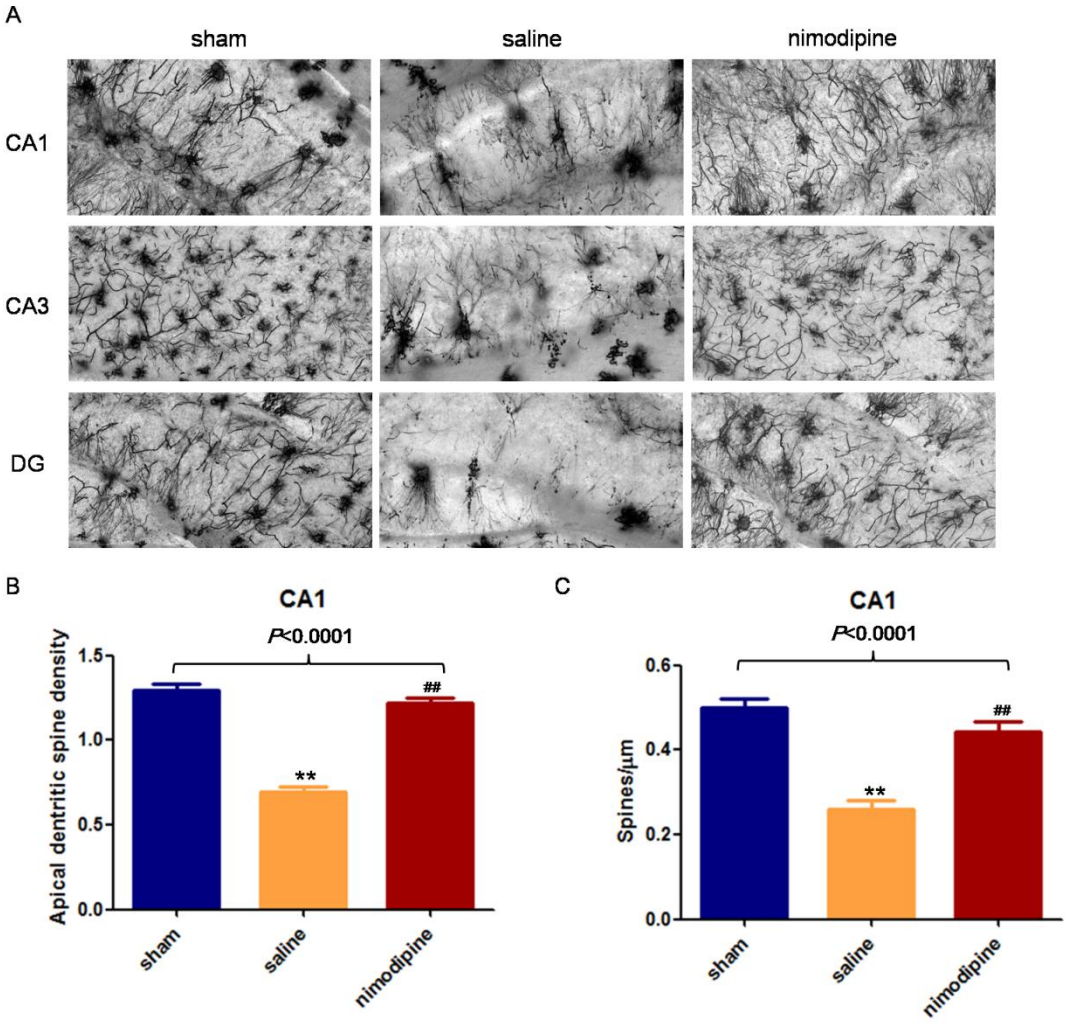

Figure 3. Detection of dentritic spines in hippocampus by Golgi staining

A: Typical section of CA1, CA3, and DG regions. B: Apical dendritic spine density in CA1 region. C: Basal spine branch density in CA1 region. Scale bar = 150 μm. $^{**}P<0.01$ vs. sham; $^{\#\#}P<0.01$ vs. saline.

Nimodipine attenuates NLRP3 inflammasome activation in the hippocampus of MCAO rats

To illustrate that NLRP3 inflammasome activation was altered in the hippocampus, expression of NLRP3 protein, cleaved caspase-1, IL-1β, and IL-18 was measured by Western blot (Figure 4). The expression of NLRP3 and cleaved caspase-1, the major components of NLRP3 inflammasome, significantly increased in the saline group compared with the sham group (both $P<0.01$). Sequentially,

expression of IL-1β and IL-18, the products of active inflammasome, also significantly increased (P<0.05 and P<0.01, respectively). The results indicated that NLRP3 inflammasome was active in the hippocampus of MCAO rats. The expression of these proteins significantly decreased in the nimodipine treatment group (all P<0.05). Nimodipine reduced expression of cleaved caspase-1 and IL-1β to the same level as the sham group; whereas the expression of NLRP3 and IL-18 were still higher than that in the sham group.

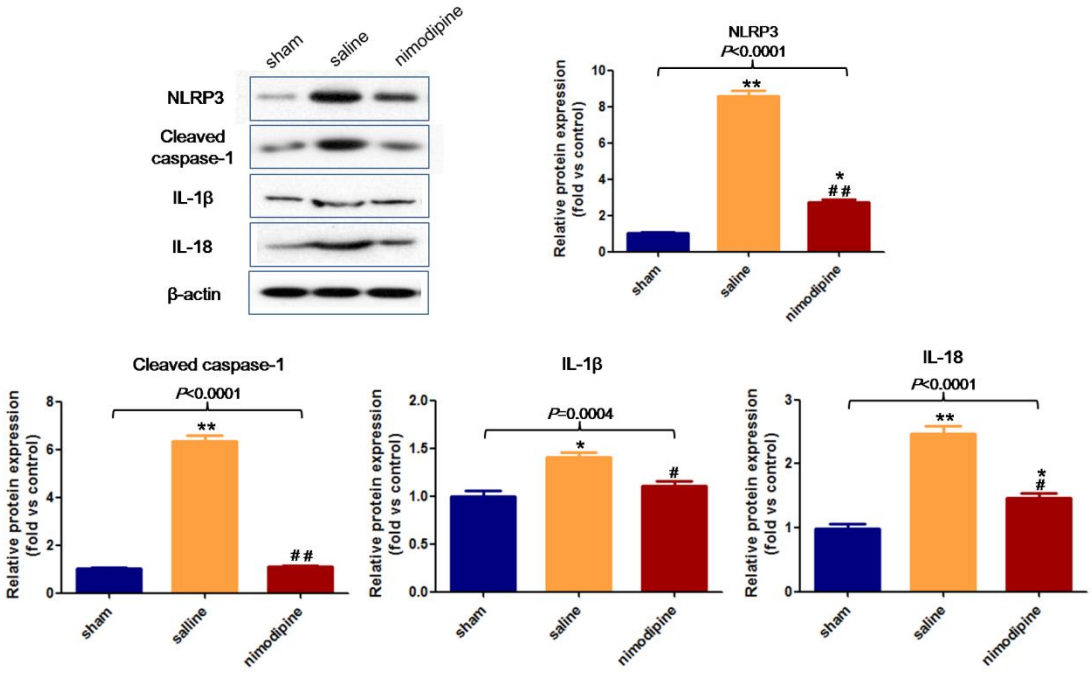

Figure 4. Detection of protein expression of NLRP3, cleaved caspase-1, IL-1β, and IL-18 by Western blot

*P<0.05 vs. sham;**P<0.01 vs. sham; $^{\#}P$<0.05 vs. saline; $^{\#\#}P$<0.01 vs. saline.

Discussion

Reperfusion injury is an important problem to be solved in the clinical treatment of cerebral ischemia. The mechanism of cerebral ischemic/reperfusion injury is very complex, including excitotoxicity, apoptosis, reactive oxygen species (ROS), inflammation, mitochondrial damage, all of which result in cell death and brain damage (3, 22). The NLRP3 inflammasome is a trigger signal of the IL-1β/IL-18 associated inflammation. In this study, we demonstrated that nimodipine treatment

improved spatial memory after cerebral ischemic/reperfusion, confirming nimodipine's neuroprotective effect. In addition, nimodipine decreased NLRP3 inflammasome activation induced by cerebral ischemic/reperfusion, indicating that nimodipine had anti-inflammatory effect.

Discrepancies exist between promising experimental animal data and unfavorable clinical application about nimodipine treatment. The results of VENUS (Very Early Nimodipine Use in Stroke) trial did not support beneficial effect of nimodipine for neurological status (23). Some other clinical studies did not support nimodipine's ability to improve functional outcome based on modified Mathew scale (24). However, those studies mainly evaluated patients' motor function and sensory function rather than spatial memory ability. Those abilities correspond to different areas of the brain. Our study showed that nimodipine treatment could improve spatial memory in rat model. This beneficial effect was also observed in a large sample size clinical study (12). Not mention that nimodipine has achieved favorable therapeutic effect in many cognitive degeneration diseases such as Alzheimer's disease (25). These studies suggest that the adaptive symptoms of nimodipine may not be to reduce infarct area or restore motor function, but to improve vascular dementia.

The neuroprotective effect of nimodipine could be attributed to attenuating both necrotic and apoptotic death (26). Our study showed consistent results that nimodipine could reduce apoptosis in hippocampus. Nimodipine is an L-type Ca^{2+} channel blocker and is crucial for Ca^{2+} homeostasis. Oxidative stress is the most direct response to ischemic/reperfusion injury, which can cause and be strengthened by Ca^{2+} overload (27). Ischemic/reperfusion injury is a stimulus of the production of mitochondrial ROS and inducer of the NLRP3 inflammasome activity. Some studies reported that mitochondrial ROS promoted the transcription of NLRP3 and pro-IL-1β rather than the activation of NLRP3 inflammasome (28). Our results showed that nimodipine could promote the production of cleaved caspase-1 and mature IL-1β, indicating that nimodipine could regulate the NLRP3 inflammasome at post-translation level. Therefore the effect of nimodipine on NLRP3 inflammasome

should be more extensive that affecting mitochondrial ROS. One possible explanation is that nimodipine might inhibit K^+ efflux, which is also a stimulus of NLRP3 inflammasome (29). Whereas Ca^{2+} antagonists can inhibit K^+ efflux is a long-known conclusion (30).

We conclude that the results of this study support the use of nimodipine after ischemic stroke to improve spatial memory. This finding would optimize the therapeutic value of nimodipine in future clinical practice.

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