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Original Article

Catalpol Alleviates Depression by Inhibiting NLRP3 Inflammasome via TLR4/MAPK/NF-Kb Pathway

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Abstract

Background: We aimed to explore catalpol and NF-k. The role of antidepressant and anti-inflammatory effects of b inhibitor in depression induced by chronic unpredictable mild stress (CUMS).

Methods: Under the guidance of Qiqihar Medical University, from January 2020 to January 2021, the weight, sucrose consumption and rest time of mice during swimming were monitored, the neurobehavioral changes of rats under CUMS were used to determine the experimental model; ELISA detection of iNOS, ROS, caspase-1, IL-1 β And IL-18 expression level; Western blotting detection of TLR4, MAPK and NF-κB expression level; LPS-induced cell model. INOS, NLRP3, caspase-1, IL-1 in RT-qPCR and ELISA detection models β And IL-18 expression level;the TLR4, MAPK and NF-κB level were detected by Western blotting.

Results: CUMS can make rats lose weight, reduce sucrose consumption rate and prolong rest time. Catapol can enhance this effect; In the depression model, ROS, NLRP3, NF-κ B and iNOS were up-regulated Catalpol group MAPK, NF-κ Reduced expression of B and TLR4; ROS, caspase-1, IL-1β, IL-18 and iNOS protein increased. Cell model group TLR4, MAPK and NF-κ. The high protein content of B decreased in catalpol group.

Conclusion: Catalpol acts as anti-depressant and anti-inflammatory molecule indepression induced by CUMS. Combination of catalpol with NF-κB inhibitor might play a role in the treatment of depression through regulating the neuroinflammation.

Keywords: Depression; Inflammasome; Therapy

Introduction

Depression is a common and serious mental disorder, which is diagnosed by several symptoms including low mood, slow thinking, reduced mental activity and physical symptoms, and sleep disorders. The disease is a major cause of disability worldwide, potentially reducing educational attainment and increasing the rates of unemployment. The incidence of depression is approximately 15-18%, thus bringing a serious burden on society (1). Although there are some medicines, they are ineffective for about 2 % of patients. In addition, they are not curative as 80%

of patients relapse after treatment. Therefore, there is an urgent need in efficient antidepressant drug (2,3).

There is a wealth of clues indicating that inflammation plays a key role in the occurrence and development of depression. In brain, activation of Nod-like receptor protein-3 (NLRP3) inflammasome and (MAPK)/Nuclear factor kappa B (NF-κB)-related pathways by inflammation plays a key role in the onset of depression (4). In addition, TLR4/MAPK/NF-κB pathway might activate NLRP3. Indeed, in osteoporosis, TLR4/MAPK/NF-κB contribute to the activation of NLRP3 inflammasome (5). Inhibition of NLRP3 and TLR4/MAPK/NF-κB efficiently alleviate neurobehavioral changes in mice models of depression (6,7), underlying the two pathways as potential drug targets for combating depression (5,8). Catalpol, an iridoid mainly found in rehmannia, has been found to exert anti-inflammatory and anti-oxidant effects (9) by inhibiting NLRP3 inflammasome (10) and TLR4/MAPK/NF-κB pathway (11). Moreover, the iridoid alleviates the neurobehavioral changes in rat model of depression (12,13).

The objective of the study was to investigate the role of Catalpol with NF-κb inhibition for improving behavior-like depression in rats under Chronic Unpredictable Mild Stress (CUMS) as experimental model of depression.

Methods

Experimental animals

Animal experiments were conducted from January 2020 to January 2021, and all animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals [National Institutes of Health (NIH), Bethesda, MD, USA].

The study was approved by the Ethics Committee of Qiqihar Medical University (NO: QMU-AECC-2021-94).

Thirty Specific-Pathogen-Free female Sprague Dawley rats (purchased from the Experimental Animal Center of Qiqihar Medical University) were exposed to light for 12 h at 25 ℃, with 35% humidity and circulated air before the experiment, and were adaptively fed with 1% sucrose solution.

Animal grouping and Chronic Unpredictable Mild Stress (CUMS)

Thirty rats were randomized into 3 groups, including model group (N=10), catalpol group (N=10) and blank group as negative control (N=10). Depression-like behaviors induced by CUMS were achieved on model group and catalpol group. The blank group hosted the rats that were not stimulated by CUMS. Unpredictable chronic stimulation was given to the rats within 3 weeks, followed by random stimulation. The stimulation was irregular and discontinuous, and repeated 2-3 times: ① The iron cage was tilted at 45 ℃ for 24 h; ② Gavage was given 20% acetic acid, with the dose of 0.1 mL/kg; ③ The time of day and night was reversed for 24 h; ④ The tail was slightly clamped at 1 cm; ⑤ The rats were fed in the moist environment for 8 h (2000 mL of water/100 g of pad); ⑥ The rats were placed in the plastic tube for 2 h; ⑦ rats were fasted for 24 h. After 5 weeks of modeling, rats in catalpol group were given intraperitoneal injection of 10 mg/kg catalpol for 4 weeks, and the model group and blank group were injected with equal volume of saline, and CUMS modeling was continued while administration.

Weight test

During the experiment, rats were fed normally from 8 am to 8 pm. The body weight of the rats in each group was measured before eating at 8 am the next day, and the change in body weight was calculated.

Sucrose preference experiment

To change the drinking habits, rats were trained for sucrose intake. To do so, the animals drunk 500ml of 1% sucrose solution within 24 h, as well as 500 ml of 1% sucrose solution and 500 ml of pure water during another 24 h. After 24-h water and food fasting, the rats were given 1000 ml

pure water and 1000ml 1% sucrose solution for 1 h (change the position of water bottle once in half an hour), and then the weight of water bottles were taken to calculate the body fluid consumption, so as to calculate the consumption rate of sucrose and water and test the weight [Sucrose preference should be used as the evaluation index. Sucrose preference (%)=sucrose water consumption/(sucrose water consumption + drinking water consumption) × 100%。]. 2 rats were excluded Judgment criteria for depressive behavior: sucrose preference is lower than 0.4 or significantly decreased compared with the control group (*P*<0 05). (1 in the model group and 1 in the catalpol group).

Forced swimming experiment

Forced swimming experiment was performed on rats in the three modeled group after the last CUMS and the next day after dosing. The rats were put into a 50*30 *60 cm transparent glass cylinder, with water at 25 ℃. The cylinder was 38 cm high, thus keeping the rats against the cylinder wall while they swam for 6 min. The accumulated inactive time was recorded after 4 min. The water was changed at intervals and the rats were dried simultaneously. The longer the immobility time is, the more severe the depression is. The immobility time of normal rats was within 30%~70% of the detection time; Judgment standard of depressive behavior: immobility time increased significantly compared with the control group (*P*<0 05).

Specimens collecting

After the animal experiments, the rats were anesthetized by intraperitoneal injection of solution containing 10% chloral hydrate (300~350 mg/kg), then 5ml blood were taken. The abdomen was cut with medical scissors along the sterilized skin. Then, the brain was extracted from the rats and put on the ice. The hippocampal tissue was stripped and placed into a frozen tube with RNAse inhibitors. The samples were stored into a -80 ℃ refrigerator. The collected blood was centrifuged for 15 min at 4 °C, 3000 r/min, and stored at -80 °C for later use.

Cell Model Construction

Hippocampal neurons of HT22 mice were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai). HT22 neurons were cultured in DMEM (Sigma) cell complete medium containing 10% fetal bovine serum (Invitrogen) and 100 U/ml penicillin/streptomycin (Sigma). The culture medium was removed, washed with phosphate buffered saline (Sigma), and the cells were digested with 0.25% EDTA-trypsin and added to the culture medium. The cells were divided into four groups, namely catalpol group, catalpol combined inhibitor intervention group, lipopolysaccharide (LPS) group and blank group. The cell density was adjusted to 5 *10^5 cells / ml, and the cells were inoculated in a 24-well plate, 600 μL / well. After 24 hours, the catalpol group (1 μg/ml lipopolysaccharide and 2.5 mg/ml catalpol) and the catalpol combined inhibitor intervention group (1 μg/ml lipopolysaccharide and 2.5 mg/ml catalpol and MAPK inhibitor SB203580 (final concentration 20 μM)), lipopolysaccharide (LPS) group (1 μg/ml lipopolysaccharide) and blank group (equal volume medium) were treated respectively. After 48 hours, it was centrifuged at 3000 r/min for 20 min, and the supernatant after centrifugation was put in a refrigerate at 80°C for later use.

ELISA test

According to the instructions of the iNOS, ROS, caspase-1, IL-1β and IL-18 ELISA kits, the expression levels of iNOS, ROS, caspase-1, IL-1β and IL-18 in the blood and cell culture supernatants of the three groups were detected. The ROS ELISA kit was from Sunberga Biotechnology Company, cat.no. SBJ-RO799. The caspase-1, IL-1β and IL-18 ELISA kits were purchased from Boster Biological Co., Ltd., cat.no. BA2220-1, EK0394 and A00124-1, respectively. iNOS was purchased from abcam, cat.no. ab253219.

RNA extraction and quantification by RT-qPCR

Mice hippocampus and total RNA in cells were

extracted by TRIZOL method. cDNA was synthesized using the reverse transcription kit (RR047A, Takara, China) following the reverse transcription conditions of 42 ℃ for 2 min, 37 ℃ for 15 min and 85 ℃ for 5 s. Then, the resulting fragment was kept at 4 ℃. qPCR amplification was performed using SYBR Green (Vazyme, Nanjing, China). The amplification conditions were as follows: 95 ℃ for 3 min, 95 ℃ for 15 s, 60 ℃ for 30 s, repeating for 40 cycles. PCR was achieved using the Applied Biosystem 2720 (Life Technologies, USA) thermocycler. The primers sequences were: NLRP3: Forward: 5'-GCTGGTCTTCAATTCCTCA-3', Reverse: 5'GGCACACGGATGAGTCTTT-3'; The GAPDH primers were: Forward: 5'-TACTAGCGGTTTTACGGGCG-3', Reverse: 5'-TCGAACAGGAGGAGCAGAGGCGA-3').
Ct averaged and $2^{-\Delta\Delta Ct}$ values were calculated and normalized to this of GAPDH as internal control. All experiments were done in triplicate and results were expressed as a mean with the standard deviation.

Western blotting analysis

The protein was separated in SDS-PAGE system according to the instructions of the existing literature (14). The protein antibodies used were as follows: *P*-NF-*κ*B antibody (diluted at 1:1000; Cell Signaling Technology, catalog number: 9936T), p-MAPK (ERK1/2) antibody (diluted at 1:1000, Cell Signaling Technology, catalog number: 5235LF), p-TLR4 antibody (diluted at 1:500; Abcam, catalog number: ab13556) and GADPH antibody (diluted at 1:1000 dilution; Abcam, catalog number: ab9485). Polyclonal goat anti-rabbit antibody (Cell Signaling Technology) was used as a secondary antibody (at room temperature for 2 h), which was imaged with ECL chemiluminescence reagent (Amersham) and detected by Western blot (Millipore).

Statistical analysis

Statistical data were analyzed with GraphPad Prism 7. Additional statistical analyses were performed with SPSS 20.0 (IBM Corp., Armonk, NY, USA). Statistical analysis of behavioral differences was performed with ANOVA and Student's t test, and PCR expression with Mann–Whitney U. Differences in protein expression in vitro were determined by ANOVA and Student's *t* test as well. *P*-values were calculated as two-tailed. Values were defined as statistically significant when $P<0.05$.

Results

Catalpol alleviates neurobehavioral changes in the rat model of depression

First, we investigated the neurobehavioral changes of rats under CUMS for confirming the experimental model of depression (Fig. 1 A). the weight of rats from the CUMS group was lower than this of rats from control group. However, the weight of rats from the CUMS group was higher when rats were treated with catalpol. Beside of the weight, we confirmed that CUMS also decreased the rate of consumption of sucrose and water as shown in Fig. 1B. In addition, catalpol increased the weight of rats under CUMS. Fig. 1C confirmed a reduced mobility for rat in the CUMS group, which was improved by catalpol as recorded by the immobility time of forced swimming.

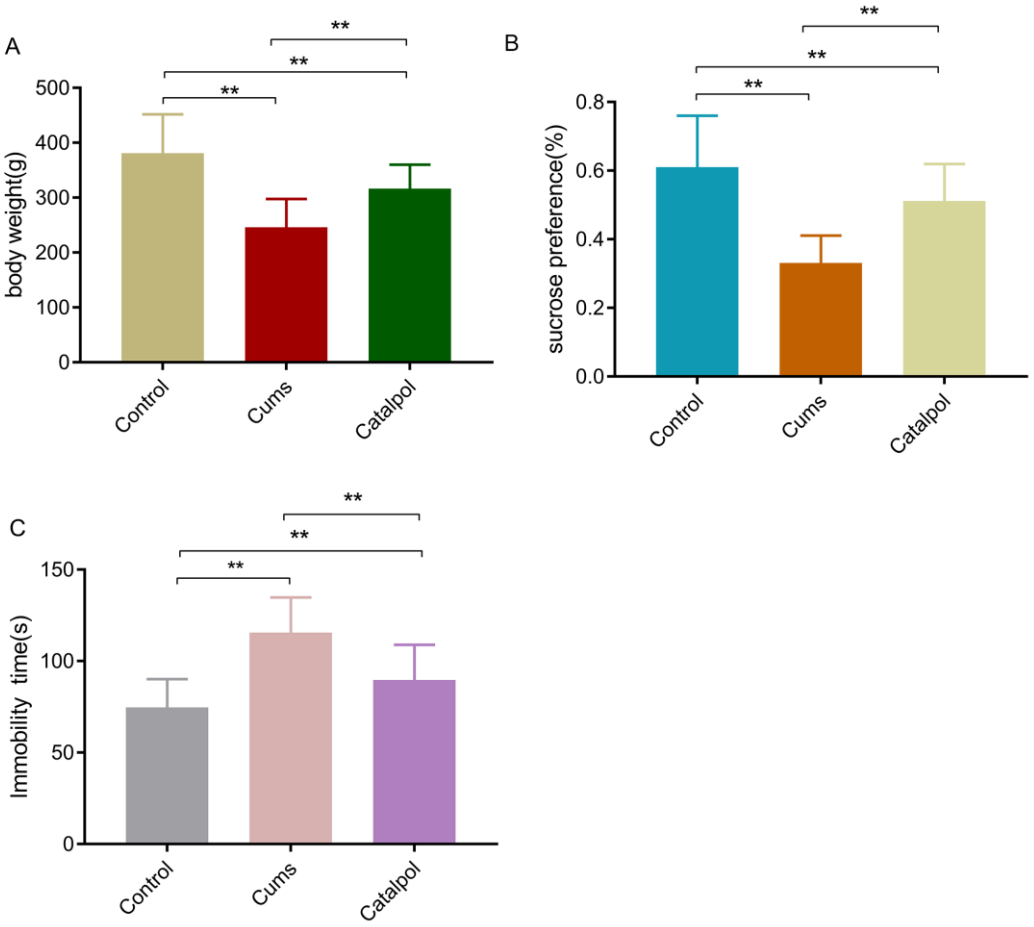

Fig. 1: Catalpol alleviates neurobehavioral changes in the CUMS model

Note: A: Changes of weight in the control (blank) group, model group and catalpol group before and after modeling and treatment; B: Changes of consumption rate of sucrose and water in blank group, model group and catalpol group before and after modeling and treatment; C: Changes of immobility time of forced swimming experiment in blank group, model group and catalpol group before and after modeling and treatment ** Compared with experimental group, $P<0.05$

Catalpol inhibits the expression levels of NLRP3 and its downstream proteins

In order to further confirm the specific role of the NLRP3 inflammasome in depression, we detected ROS, NLRP3 Expression levels of inflammasome and downstream molecules of caspase-1, IL-1β, IL-18 and NF-ϰB iNOS in rat neuronal depression model. Fig. 2A shows that the expressions of iNOS, ROS, caspase-1 IL-1β and IL-18 in the model group were up-regulated, and the catalpol administration group was relieved. Fig. 2B shows that the expression of NLRP3 and inflammasome in the model group was up-regulated, and the catalpol administration group was relieved.

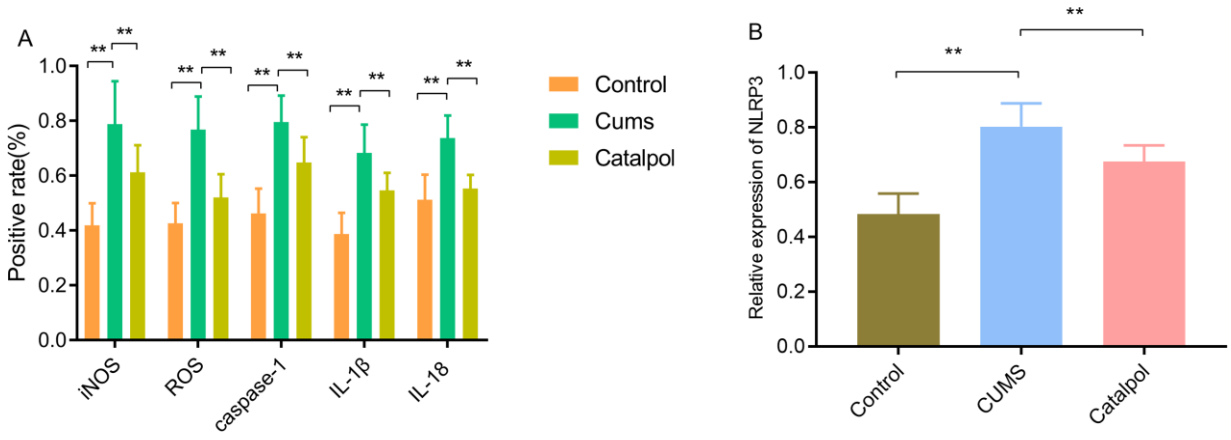

Fig. 2: NLRP3 inflammasome and downstream proteins were upregulated in depressed rats

Note: A: ELISA was used to detect the expression levels of iNOS, ROS, caspase-1, IL-1β, and IL-18 in depression model B: qRT-PCR was used to detect the expression level of NLRP3 inflammasome in depression model. ** Compared with the experimental group, $P<0.05$

Catalpol regulates NLRP3 bodies through the TLR4/MAPK/NF-κB pathway

In order to confirm the specific mechanism of the role of MAPK/NF-κB pathway in catalpol regulating NLRP3 inflammasome in the treatment of depression, we analysis the expression levels of TLR4, MAPK and NF-κB in mouse neuronal depression model. Figures 3A and 3B show that compared with the model group, the protein expressions of MAPK, NF-κB and TLR4 in the hippocampus of the catalpol group were relatively decreased. The above results further confirmed that catalpol regulates the expression of NLRP3 inflammasome through the MAPK/NF-κB pathway, thereby exerting a therapeutic effect on depression.

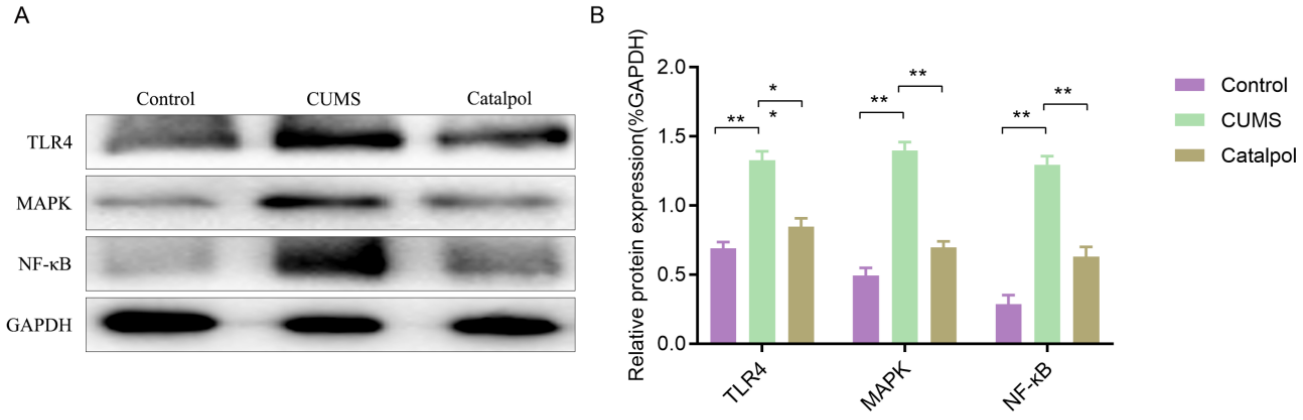

Fig. 3: Catalpol regulates NLRP3 bodies through TLR4/MAPK/NF-κB pathway

A: Western blotting was used to detect the protein levels of TLR4, MAPK, NF-κB and GADPH in blank group, drug intervention group and model group. B: The intensity of each band was quantified with ImageJ and normalized to GAPDH, then to the blank group. **$P<0.05$

Catalpol alleviates neuroinflammation by regulating NLRP3 and downstream pathways through TLR4/MAPK/NF-κB pathway

In order to confirm whether neuroinflammation plays a role in catalpol regulating NLRP3 inflammasome in the treatment of depression and the specific role of TLR4/MAPK/NF-κB pathway, we analyzed the expression levels of ROS, NF-κB downstream iNOS, NLRP3 inflammasome and downstream caspase-1, IL-1β, IL-18 in LPS-treated HT22 cells mimicking hippocampal neuroinflammation model. Fig. 4A shows that after LPS treatment, the expression of ROS, caspase-1, IL-1β, IL-18 and iNOS proteins in the model group increased. Compared with the model group, the protein expression of catalpol group was relatively decreased, and the protein expression of catalpol combined inhibitor group was further decreased, indicating that catalpol inhibits the expression levels of ROS, caspase-1 and downstream inflammation through the TLR4/MAPK/NF-κB pathway. Fig. 4B shows the relative increase in NLRP3 mRNA expression in the model group after LPS treatment. Compared with the model group, the NLRP3 mRNA expression in the catalpol group was relatively decreased, and the NLRP3 mRNA expression in the catalpol combined inhibitor group was further decreased.

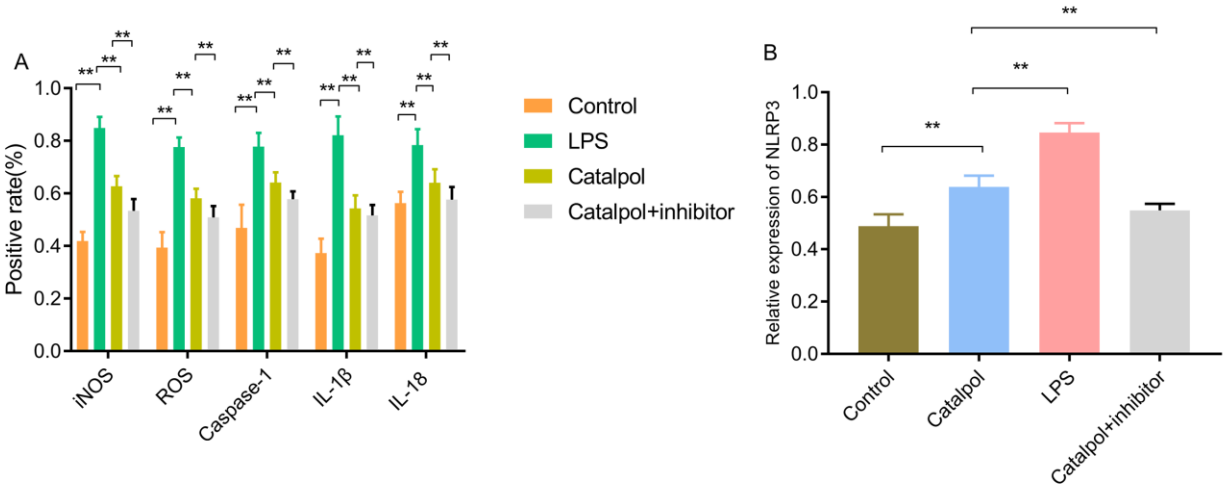

Fig. 4: Catalpol alleviates neuroinflammation by regulating NLRP3 and downstream pathways through TLR4/MAPK/NF-κB pathway

A: The expression levels of iNOS, ROS, caspase-1 IL-1β and IL-18 in the above four groups were detected by ELISA. B: The expression levels NLRP3 inflammasome was detected by qRT-PCR in the drug intervention group, the drug + inhibitor group, the model group and the blank group

Catalpol regulates the activation of the NLRP3 inflammasome through the TLR4/MAPK/NF-κB pathway

Further analysis whether catalpol passes TLR4/MAPK/NF- κ The B pathway regulates the body of NLRP3.

Fig. 5A and 5B showed that the high protein contents of TLR4, MAPK and NF-κB induced by LPS treatment in the model group were diminished in the catalpol group. The inhibitory effect of catalpol on the three proteins was potentiated by BAY 117082.

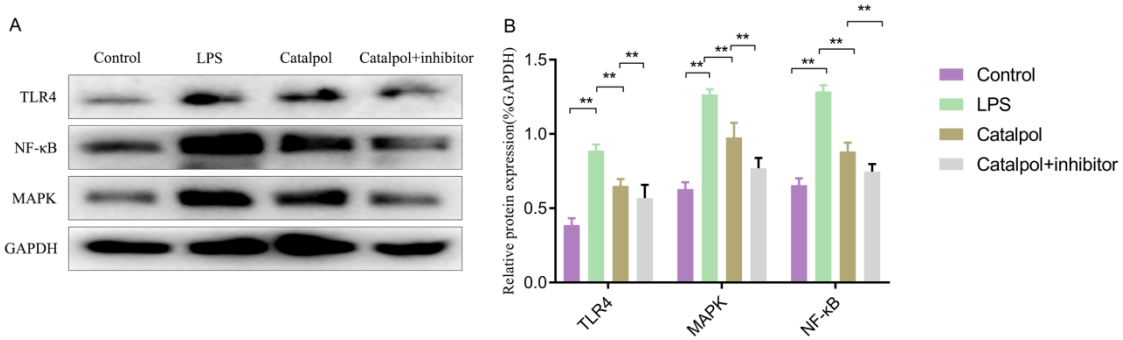

Fig. 5: Catalpol alleviates neuroinflammation through TLR4/MAPK/NF-κB pathway

Note: A: Western blotting was used to detect the protein levels of TLR4, MAPK, NF-κB and GADPH in blank group, drug intervention group, inhibitor group and model group. B: The intensity of each band was quantified with ImageJ and normalized to GAPDH, then to the blank group. ***P*<0.05

Discussion

Evidence for the role of neuroinflammation in the development of depression and thereby, as a therapeutic target against depression, is growing (15). Indeed, it has been shown an increase in the NLRP3 inflammasome which, was followed by an increase of plasma level of IL-1β, and IL-18 and blood monocytes in depressed patients (16,17). Catalpol played a role in depression in rat models by regulating the PI3K/AKT/Nrf2/HO-1 pathway, further confirming that catalpol can improve neuroinflammation and alleviate the behavioral change of depression models (18).

Indeed, Rethorst et al (19) found a strong positive association between NLRP3 and IL-1β in the serum of depressed patients, and this correlation was proportional with the severity of depression. NLRP3 induced the expression of IL-1β (5). Inactivation of NLRP3 reduces the peripheral blood IL-1β levels and protects rats against depression-like phenotype caused by CUMS. Therefore, IL-1β expression has been proposed as a marker for evaluating the efficacy of antidepressant treatment. Likewise, IL-18, a downstream target of NLRP3, is positively associated with the severity of depressive symptoms in patients with schizophrenia (20). In addition, the rise of IL-18 expression was correlated with the change in hippocampal volume of patients.

In this study, we confirmed activation of NLRP3 inflammasome including the increased expression of NLRP3, IL-1β and IL-18 in the hippocampus of rats living depression-like behaviors caused by CUMS. In addition, we found that catalpol inhibits the inflammatory pathway, an effect that was associated with improved neurobehavior. Catalpol can reduce this increase to a certain extent, confirming that this pathway is involved in the pathophysiological process of depression, and catalpol may alleviate behavioral changes in patients with depression by regulating the expression level of this pathway. In depression, NF-κB inhibited by siRNA could somewhat alleviate neurobehavioral changes in animal model of depression (21). Feng et al (22) have also confirmed that chronic stress can upregulate NF-κB expression. In this report, we found that combination of NF-κB inhibitor with catalpol efficiently alleviate neurobehavioral changes induced by CUMS.. The TLR4/NF-κB pathway is expressed in the process of depression model, and there are similar changes in neuroinflammation model, which is also confirmed by our research results (23). However, this study also revealed that other MAPK-related responses such as p38MAPK/JNK also play a regulatory role in it. The specific role of MAPK-related pathways in depression models and whether the regulation of catalpol is related to other pathways may also be further discussed.

There is a wealth of studies showing the key role played by TLR4/MAPK/ NF-κB in the activation of NLRP3 inflammasome (24). This link has

been well studied in osteoporosis. In order to further explore whether catalpol can regulate the regulation of TLR4/MAPK/NF-κB pathway to relieve neuroinflammation and regulate the expression level of NLRP3 inflammasome, we used MAPK inhibitor combined with catalpol for drug intervention to detect the levels of NLRP3 and other related factors. The results showed that TLR4/MAPK/NF-κB alleviated neuroinflammation through the NLRP3 inflammasome. It was speculated that catalpol alleviated neuroinflammation through the intervention of this pathway, thereby improving the behavioral changes of the depression rat model. An et al (25) confirmed the regulation of ROS and MAPK/NF-κB pathway on NLRP3 inflammasome, inhibiting ROS/MAPK/NF-κB expression can inhibit the activation of NLRP3 inflammasome, and thus the activity of osteoclasts. Inhibition of TLR4/NF-κB can inhibit the activation of NLRP3 inflammasome and alleviate ischemia-reperfusion injury (26). Inhibiting NF-κB/COX-2/PG could also inhibit the expression level of NLRP3, thereby improving dysmenorrhea symptoms (27). Therefore, multiple pathways are also involved in the regulation of NLRP3 inflammasome. Further studies are needed to confirm the role of other pathways in the pathophysiology of depression.

Conclusion

Our study highlighted the key role of TLR4/MAPK/NF-κB in the pathophysiology of depression by triggering NLRP3 inflammasome in rat model of depression caused by CUMS. Catalpol plays a protective role by inhibiting ROS generation and reducing the expression of NLRP3 inflammasome, which is synergized by NF-κB inhibitor. Future therapies combating depression may consider the drug targeting of TLR4/MAPK/NF-κB.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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