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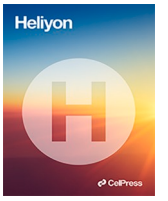

Novel oxicam nonsteroidal compound XK01 attenuates inflammation by suppressing the NF-κB and MAPK pathway in RAW264.7 macrophages

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ABSTRACT

Traditional non-steroidal anti-inflammatory drugs (NSAIDs) show serious adverse effects during clinical use, which limits their usage. Oxicams (e.g., piroxicam, meloxicam) are widely used as NSAIDs. However, selectivity to cyclooxygenase (COX) 2 may cause cardiovascular problems considering the long-term use of the drugs. Therefore, it is important to develop new non-steroidal compounds as anti-inflammatory drugs. In the present study, we evaluated the anti-inflammatory activity of a newly developed nonsteroidal drug XK01. Our data showed that XK01 reduced the contents of nitric oxide (NO) and reactive oxygen species (ROS)and inhibited the transcription levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β in a dose-dependent manner in lipopolysaccharide (LPS)-stimulated mouse RAW264.7 macrophages. XK01 showed no significant inhibitory effect on COX-1, but inhibited the expression of COX-2. At molecular level, XK01 prevented the translocation of p65 protein from the cytoplasm to the nucleus and inhibited the phosphorylation of p65, IκB, and MAPKs proteins. And high concentration of XK01 also inhibited the phosphorylation of JNK, p38 and ERK, showing stronger effect than that of meloxicam. In addition, the anti-inflammatory activity of XK01 was further validated in Xylene-induced mouse ear swelling model. Thus, this study verified that XK01 inhibits the expression of inflammatory mediators and COX-2, and exhibits potential anti-inflammatory effects via suppressing the NF-κB and MAPK pathway.

1. Introduction

Inflammation is a complex reaction of the immune system induced by stimuli, which provides self-protection for the host [1]. Infected tissues will activate phagocytosis, macrophages and increase the level of inflammatory factors, leading to damage to the

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organism.

Macrophages are cells differentiated from monocytes with phagocytosis, immune response, regulation of tissue regeneration, and removal of senescent cells. Macrophages play an important role in maintaining the homeostasis of the internal environment. Under the stimulation of pathogens, macrophages rapidly secrete active substances such as proteases and cytokines, phagocytose and digest cell debris or pathogens, and further activate immune cells or lymphocytes to respond to pathogens [2,3]. Therefore, macrophages are often used as cellular models to explore the immune and inflammatory responses of the body.

Among the stimuli that cause inflammation, lipopolysaccharides (LPS) are components of the outer membrane of the cell wall of Gram-negative bacilli, which act as natural immune-inducing factors that activate the immune response and induce infectious inflammation. Lipopolysaccharides (LPS) can activate the immune response and induce inflammation. LPS forms a complex with LPS binding protein (LBP) and pattern recognition receptors such as CD14 and Toll-like receptor 4 on the cell membrane surface of macrophages [4], which activates the adaptor protein myeloid differentiation protein (MyD88) and related kinases. Then turn on the NF-κB signaling pathway to induce inflammatory response, regulating the synthesis and release of inflammatory factors including tumor necrosis factor (TNF), interleukins (ILs), nitric oxide (NO), cyclooxygenases (COX) and prostaglandin PGE2 [5,6], causing symptoms such as fever, endothelial damage, capillary leakage, coagulation and microcirculation disturbances [7].

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs with anti-inflammatory, antipyretic and analgesic effects [8]. When inflammation occurs, NSAIDs exert anti-inflammatory effect via blocking the synthesis of COX, thus the COX fails to catalyze the synthesis of cyclic endoperoxides from arachidonic acid (AA) to form PGE2. Traditional NSAIDs, such as diclofenac, piroxicam, and indomethacin are mostly non-selective COX inhibitors, which can inhibit COX-1 and COX-2, showing a stronger inhibitory effect on COX-1. Meloxicam, an important member of NSAIDs, exhibits good anti-inflammatory effects, and plays an increasingly important role in clinical practice because it preferentially inhibits COX-2 more than COX-1. However, long-term use of this drug is a challenge to cardiovascular health. And Meloxicam blocks the production of COX-1-derived prostaglandins (PGs) and disrupts the protective effect of COX-1 on the gastric mucosa, causing gastrointestinal side effects [9,10]. Therefore, the search for NSAIDs with strong efficacy and low side effects remains a key research priority both clinically and in terms of socio-economic benefits.

Studies have demonstrated that activation of NF-κB promotes the expression of COX-2 [11]. In contrast, MAPK family members induce COX-2 gene expression in various cell types such as macrophages, cardiomyocytes, human umbilical vein endothelial cells (HUVECs), and smooth muscle cells [12,13], and MAPKs regulate the cellularity of macrophages during stimulation [14,15].

In summary, the aim of the present study was to investigate the anti-inflammatory activity and mechanism of the novel xylocaine NSAID, XK01, which is a novel compound modelled on the structure of the xylocaine NSAIDs. We evaluated the anti-inflammatory effects of XK01 on LPS-stimulated mouse peritoneal monocyte macrophages, RAW264.7 cells, and an animal model was established to verify the in vitro results through in vivo experiments. This will provide a theoretical basis for the further development of novel non-steroidal anti-inflammatory drugs, and is expected to provide a better choice of anti-inflammatory drugs for clinical use.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and PBS were purchased from Hyclone (Logan, Utah). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY, USA). LPS and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich Co. (St. Louis, Mo, USA). Enzyme-linked immunosorbent assay (ELISA) kits for measuring IL-6,TNF-α and PGE2 mouse ELISA kit was purchased from Elabscience (Wuhan, China). ROS kit and NO detection kit were purchased from Shanghai Beyotime (Shanghai, China). RNA extraction kit and RNA reverse transcription kit were purchased from Vazyme Biotech Co. (Nanjing, China). RT-PCR Mix and ultra-sensitive ECL chemiluminescence kit were acquired from BioRad Laboratories (Hercules, CA, USA). Antibodies for P38, p-P38, JNK, *p*-JNK were purchased from Signalway Antibody (Califorlia,US). Antibodies for p–NF–κB, NF-κB, ERK and p-ERK were purchased from Cell Signaling Technology (Beverly, MA, US) (Table 1). XK01 was synthesised by the the laboratory of

Table 1
The catalog numbers of the antibodies.

Name	Brand	Catalog Number
p-NF-κB	Cell Signaling Technology (Beverly, MA, US)	# 3033S
NF-κB	Cell Signaling Technology (Beverly, MA, US)	# 8242S
ERK	Cell Signaling Technology (Beverly, MA, US)	# 4695S
p-ERK	Cell Signaling Technology (Beverly, MA, US)	# 4370S
COX-1	HuaBio	ET1610-98
COX-2	Proteintech	12375-1-AP
p-IKBα	Zen-Bioscience (Chengdu, China)	# 340776
p38	Signalway Antibody (Califorlia,US)	# 48644
p-p38	Signalway Antibody (Califorlia,US)	# 12322
JNK	Signalway Antibody (Califorlia,US)	# 48615
p-JNK	Signalway Antibody (Califorlia,US)	#11504
Histon H3	Signalway Antibody (Califorlia,US)	#32667
α-tubulin	Signalway Antibody (Califorlia,US)	#37981
β-Action	Signalway Antibody (Califorlia,US)	#38074

Department of Chemical Engineering, Hunan Normal University (Fig. 1).

2.2. Cell culture

RAW264.7 cells were purchased from the Cell Resource Center, Shanghai Academy of Life Sciences, Chinese Academy of Sciences. The cells were maintained in a high-glucose DMEM supplemented with 10 % FBS and 1 % of an antibiotic mix, at 37 °C in a humidified incubator with 5 % CO_2.

2.3. Cell viability assay

MTT assay was conducted to evaluate the cytotoxicity of LPS, Meloxicam (Melo, positive drug) and XK01. RAW264.7 cells were seeded in a 96-well plate and cultured for 24 h. After 24 h, different concentrations of XK01 or meloxicam drugs were added and treated for 24 h.After the drug effect was over, the culture medium was discarded and 50 μl of prepared MTT (2 μg/ml) solution was added to each well, and incubated for 5 h in an incubator at 37 °C. After and incubating for 5 h, the medium was removed and 150 μL of dimethyl sulfoxide was added to each well. To fully dissolve the formazan crystals, the plate was covered with silver paper and shaked on a shaker for 20 min. The absorbance (A) value at 490 nm was read using a microplate reader. The data from three independent experiments was processed by Prism (GraphPad Software, San Diego, CA) program.

2.4. NO assay

After treatment, 60 μl of the sample was taken directly in the cell culture supernatant and added to another 96-well plate, and the sequential addition of the Griess Reagent I (50 μL per well) and Griess Reagent II (50 μL per well) were added to the cells, respectively and incubated for 3 min in dark. Then the absorbance (A) at 540 nm wavelength was read using a multifunctional microplate reader to measure the content of NO.

2.5. ROS analysis

The cells were seeded in a 24-well plate and cultured for 24 h. Then, cells were culture in serum-free medium for 12 h followed by treatment with different concentrations of drugs, and LPS inducers were added. After 24 h, ROS fluorescent probe 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was added and incubated for 30 min. Wash the cells with serum free medium to remove the remaining fluorescent probes. The fluorescence intensity was observed under a fluorescence microscope)and quantified using Image J 15.3 software.

2.6. ELISA assay

After treatment, cells were collected and lysed by repeated freezing and thawing. The supernatant was collected after centrifuge. Levels of TNF-α, IL-1β and IL-6 were measured using ELISA kits according to the manufacturer's instructions.

2.7. Real-time PCR

RNA was extracted by using the TRI reagent (Thermo Fisher Scientific, Waltham, MA). cDNA was generated by using the RNA reverse transcription kit. Quantitative RT-PCR was performed in a CFX96 real-time system (Bio-Rad, Hercules, CA). The specific sense and antisense primers were listed in Table 2. The PCR reaction system containing 12.5 μL SYBR Green PCR master mix, 10.5 μL of 1 μM primer stock and 2 μL of cDNA. The primers used in this study are listed in Table 2.

2.8. Western blots

Extraction of cytoplasmic and cytosolic proteins: Pour off the cell culture solution in the six-well plate after administration, wash the cells twice with 1 × PBS and then aspirate the liquid in the plate as much as possible. Separate the cytosolic proteins from the cytoplasm according to the instructions of the Cytosolic and Cytoplasmic Protein Extraction Kit, and obtain the samples to be stored at 4 °C in the refrigerator.

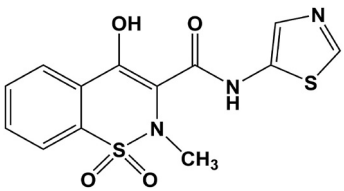

Fig. 1. XK01 structure.

Table 2
Primers for real-time PCR.

Name	Forward	Reverse
GAPDH	ACCCCAGCAAGGACACTGAGCAAG	GGCCCCTCCTGTTATTATGGGGGT
TNF-α	CCCTCCTGGCCAACGGCATG	TCGGGGCAGCCTTGTCCCTT
IL-1β	GCCTCGTGCTGTCGGACCCATAT	TCCTTTGAGGCCCAAGGCCACA
IL-6	TGGGACTGATGCTGGTGACA	ACAGGTCTGTTGGGAGTGGT
COX-1	GCCCTTCAATGAATACCGAAAG	GGGTAGAACTCTAAAGCATCGA
COX-2	ATTCCAAACCAGCAGACTCATA	CTTGAGTTTGAAGTGGTAACCG
PGE_2	TCTCATCGCACTGGCACTGTTG	AGGCAGGTTCCCAGCAGGTC

After treatment, cells were collected and washed with cold PBS. Cell pellets were resuspended in RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM sodium chloride, 1 % NP-40, 0.2 % SDS, 0.5 % sodium deoxycholate, 0.1 mM EDTA and 1 % protease and phosphatase inhibitors (Sigma-Aldrich). Lysates were centrifuged and supernatants were collected. Cell lysates (25 μg) were separated by 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P membranes; Millipore, Billerica, MA, USA). Membranes were blocked with blocking buffer (5 % skim milk, 0.1 % Tween-20 in PBS) for 1 h at room temperature. After incubation with primary antibodies overnight at 4 °C, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA) with the ODYSSEY Fc, Dual-Mode Imaging system (Li-COR, Lincoln, NE).

2.9. Xylene-induced mouse ear swelling model

ICR mice were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, Hunan, China). These experiments were consistent with the guidelines of the Institutional Animal Care and Use Committee at Hunan Normal University. Mice were housed under sterile conditions with access to food and water. Mice were randomly divided into 5 groups with 5 mice in each group, namely model group (normal saline), positive control group (Melo 6.0 mg/kg), XK01-LD (1.5 mg/kg), XK01-MD (3.0 mg/kg), XK01-HD (6.0 mg/kg). Each group of drugs was diluted with normal saline to the desired concentration. The mice was treated via gavage, the administration volume was 0.2 mL/20 g, once a day, and each group was administered continuously for 10 days. Referring to the method in Ref. [16], 1 h after the last administration, round ear pieces were made and weighed to calculate the degree of swelling and its inhibition ratio to swelling, and the tissue of the ear pieces was analyzed by H&E staining and IHC staining.

2.10. Statistical analysis

Data were expressed as means ± SD and were analyzed by two-tailed t-tests and two-way ANOVA. Data were given with 95 % confidence intervals and were reported with corresponding P values (*P < 0.05, **P < 0.01, ***P < 0.001). GraphPad Prism 6 and SPSS 13.0 were used for all statistical analysis.

3. Results

3.1. XK01 showed low cytotoxic effects on RAW264.7 mouse macrophages cell lines

We firstly investigated the cytotoxic effect of XK01, Melo and LPS on cell viability of RAW 264.7 mouse macrophages. The results showed that XK01 had essentially no effect on cell viability at a concentration of 25 μg/ml. As the concentration of XK01 gradually increased, the cell viability gradually decreased, especially at a concentration of 200 μg/ml, the cell viability decreased significantly. (*P < 0.05 or ***P < 0.001)(Fig. 2a). The positive control drug Melo showed similar cytotoxic effects as XK01 (Fig. 2b). Moreover, LPS

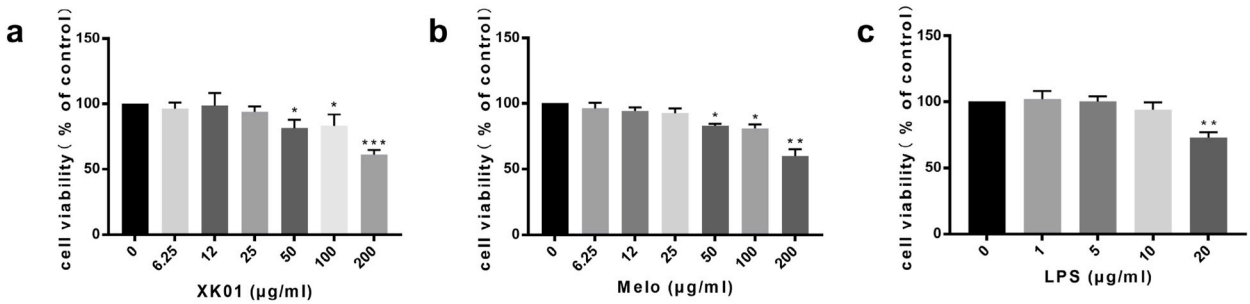

Fig. 2. Cytotoxic effects of XK01 on RAW264.7 mouse macrophages cell lines. RAW264.7 cells were treated with XK01 (a), Melo (b) or LPS (c) at various concentrations as indicated. MTT colorimetric assay was used to determine the cell viability. Graphs show mean ± SEM of at least three independent experiments. *P < 0.05. **P < 0.01, ***P < 0.001 obtained from Student's t-test.

treatment with the indicated concentrations decreased the cell viability of the mouse macrophages, with essentially no difference in cell viability from normal controls at 10 μg/ml ($P > 0.05$), and significantly inhibited cell proliferation when the concentration was increased to 20 μg/ml (Fig. 2c, $^{**}P < 0.01$). Based on the results, a concentration of 100 μg/mL of XK01 or Melo was used as the maximum concentration in the subsequent experiments.10 μg/ml was used as the induction concentration for the LPS-induced inflammation model.

3.2. XK01 suppressed LPS induced ROS and NO production in RAW264.7 macrophages

The effects of XK01 on NO production in the LPS-induced RAW264.7 cells were examined. The results showed that after LPS treatment, the NO level was significantly increased in a dose-dependent manner (Fig. 3a, $^{**}P < 0.01$ or $^{***}P < 0.001$). Pretreatment with XK01 significantly reduced the NO production in the LPS-stimulated macrophages, and a high concentration (100 μg/mL) of XK01 reduced the NO production to 11.75 ± 0.07 μM, which was similar to that of the Melo (Fig. 3b). The effects of XK01 on LPS-induced ROS production were also detected. As shown in Fig. 3c, different concentrations of XK01 (6.25 μg/mL, 25 μg/mL, 100 μg/mL) as well 100 μg/mL of Melo significantly inhibited the production of ROS in LPS-induced RAW264.7 cells (Fig. 3c). The inhibitory effect of XK01 on ROS production was confirmed through observation of fluorescent signals using DCFH-DA as a probe. As shown in Fig. 3d, no fluorescence was observed in the normal RAW264.7 cells. The fluorescence intensity was significantly increased when treated with LPS, which was gradually attenuated with the increasing concentration of XK01.

3.3. XK01 inhibited LPS-induced inflammatory cytokines secretion in RAW264.7 macrophages

We investigated the effects of XK01 on the mRNA expression of inflammatory cytokines in the LPS-induced macrophages as well levels of TNF-α and IL-1β in the cell culture supernatant. The transcriptional expression of TNF-α, IL-1β and IL-6 mRNA was significantly increased when the macrophages were treated with LPS alone when compared to the untreated cells. Pretreatment of XK01 or Melo significantly inhibited the mRNA expressions of the above mentioned inflammatory factors (Fig. 4a–c). The ELISA assay showed that LPS induced the secretion of TNF-α and IL-1β, which were also significantly decreased in the XK01-pretreated cells (Fig. 4d–e). The experimental results indicated that the new compound XK01 had a good inhibitory effect on the inflammatory cytokines release in macrophages. The inhibitory effects of high concentration XK01 was similar to that of 100 μg/mL of positive control Melo.

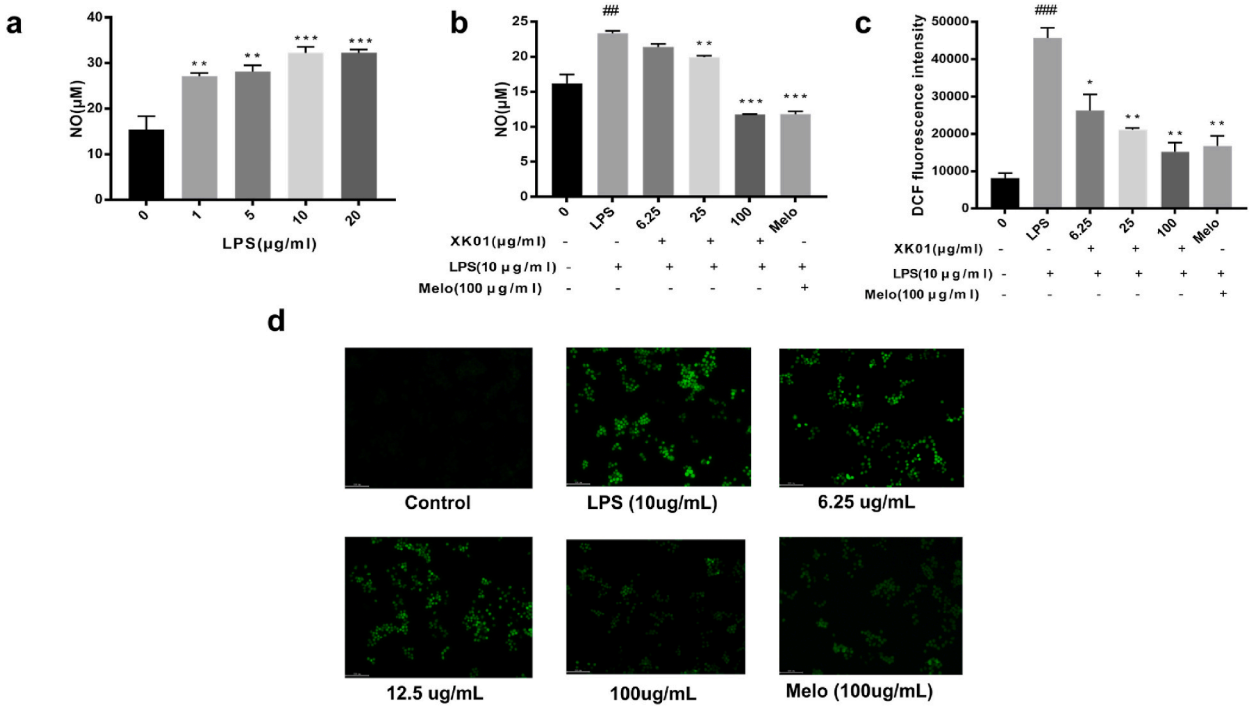

Fig. 3. XK01 inhibited NO and ROS production in LPS-activated RAW264.7 mouse macrophages cell lines. RAW264.7 cells were pre-treated with the indicated concentrations of XK01 or 100 μg/mL Melo followed by LPS stimulation. (a and b): Levels of NO were measured; c: Fluorescence intensity of ROS was quantified; d: Representative images of fluorescent signal for ROS observed under microscope. Graphs show mean ± SEM of at least three independent experiments. $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ obtained from Student's t-test.

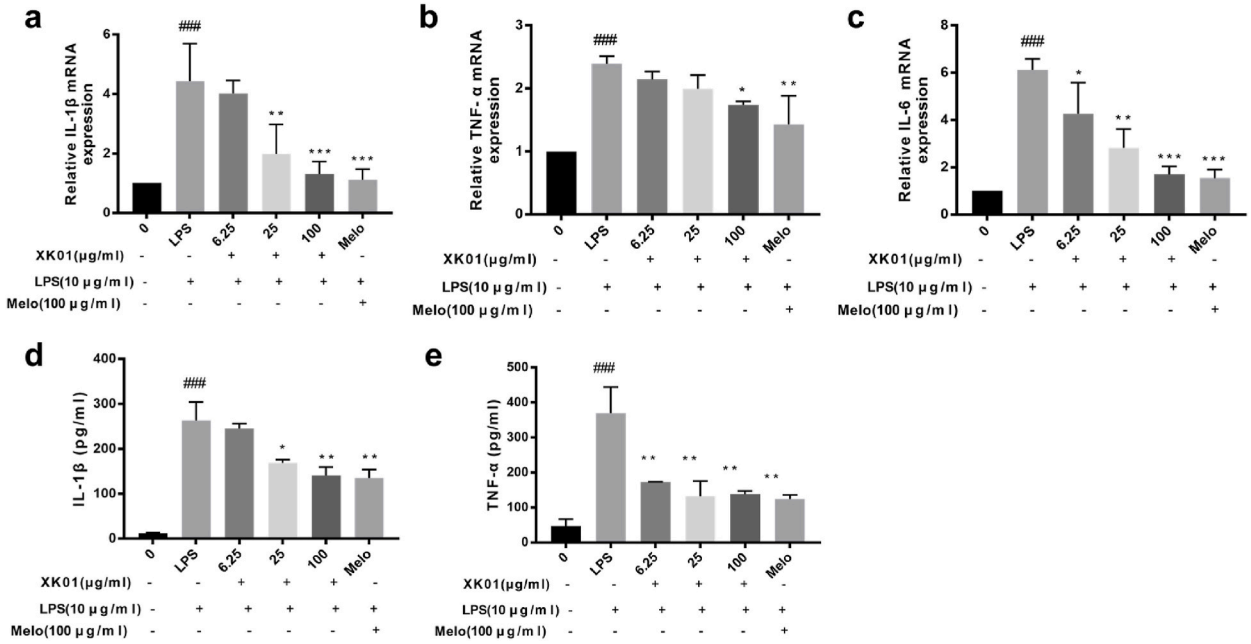

Fig. 4. XK01 inhibits proinflammatory cytokine production in LPS-activated RAW264.7 mouse macrophages. RAW264.7 cells were pretreated with Melo and different concentrations of XK01 and then stimulated with LPS (10 μg/mL). The transcription of inflammatory cytokines IL-1β (a), TNF-α (b) and IL-6 (c) RNA were detected by real-time quantitative PCR, and the pro-inflammatory cytokines IL-1β (d) and TNF-α (e) in conditioned medium were measured by ELISA. Graphs show mean ± SEM of at least three independent experiments. *P < 0.05. **P < 0.01, ***P < 0.001, $^{###}$P < 0.001 from Student's t-test.

3.4. XK01 inhibited the expression of cyclooxygenase 2 induced by LPS in RAW264.7 macrophages

The expression of COX-1 and COX-2 mRNA and protein as well the PGE2 mRNA were evaluated in the LPS-induced RAW264.7 macrophages. After LPS stimulation, the expression levels of COX-2 mRNA as well the protein were significantly up-regulated, which were attenuated by pretreatment with XK01 or Melo (Fig. 5a, e and f). However, the expression of COX-1 showed no significant difference between the treatment groups and the normal control group (Fig. 5b and c), which indicated that LPS, XK01 or Melo did not inhibit the COX-1 expression in the macrophages (Fig. 5a–c). In addition, LPS stimulated the mRNA expression of PGE2, a target of COX; pretreatment with XK01 or Melo significantly decreased the PGE2 mRNA expression, and the inhibitory effects of 100 μg/mL XK01 and Melo were similar (Fig. 5d). Similar results were also observed by using immunofluorescence assay. As shown in Fig. 5g, the COX-2 signal was increased in LPS-induced cells, which was attenuated in the XK01 or Melo-pretreated cells. These results indicate that the new compound XK01 exerts the anti-inflammatory effect at least partially via inhibition of COX-2, and it might be a selective COX-2 inhibitor.

3.5. XK01 inhibited the activation of NF-κB induced by LPS in RAW264.7 macrophages

We evaluated the effects of XK01 pretreatment on NF-κB activation in LPS-induced macrophage cell lines. The results showed that XK01 intervention decreased the phosphorylation of p65 and IκBα induced by LPS in RAW264.7 cells (Fig. 6a–c). Moreover, LPS induced the nuclear p65 and suppressed the cytosolic levels in the macrophages, which means a large amount of p65 protein in the cytoplasm entered the nucleus after LPS stimulation. Pretreatment with a medium (25 μg/mL) or high concentration (100 μg/mL) of XK01 inhibited the nuclear translocation of the cytoplasmic p65 (Fig. 6d–f). IFA assay confirmed that translocation of NF-κB p65 from the cytoplasm to the nucleus was inhibited by pretreatment of XK01 or Melo in LPS-induced RAW264.7 macrophages (Fig. 6g).

3.6. XK01 suppressed LPS-induced MAPK pathway

The mitogen-activated protein kinase (MAPK) family is widely involved in cell differentiation, metastasis, and inflammation. After LPS stimulation, the phosphorylation levels of ERK1/2, JNK and p38 in the MAPK pathway were significantly increased (Fig. 7a–d). After cells were pretreated with XK01, a medium (25 μg/mL) or high concentration (100 μg/mL) of XK01 inhibited the phosphorylation of ERK1/2, JNK and p38 induced by LPS, displaying stronger inhibitory effects on *p*-ERK1/2 and *p*-JNK at the high concentration (Fig. 7a–d).

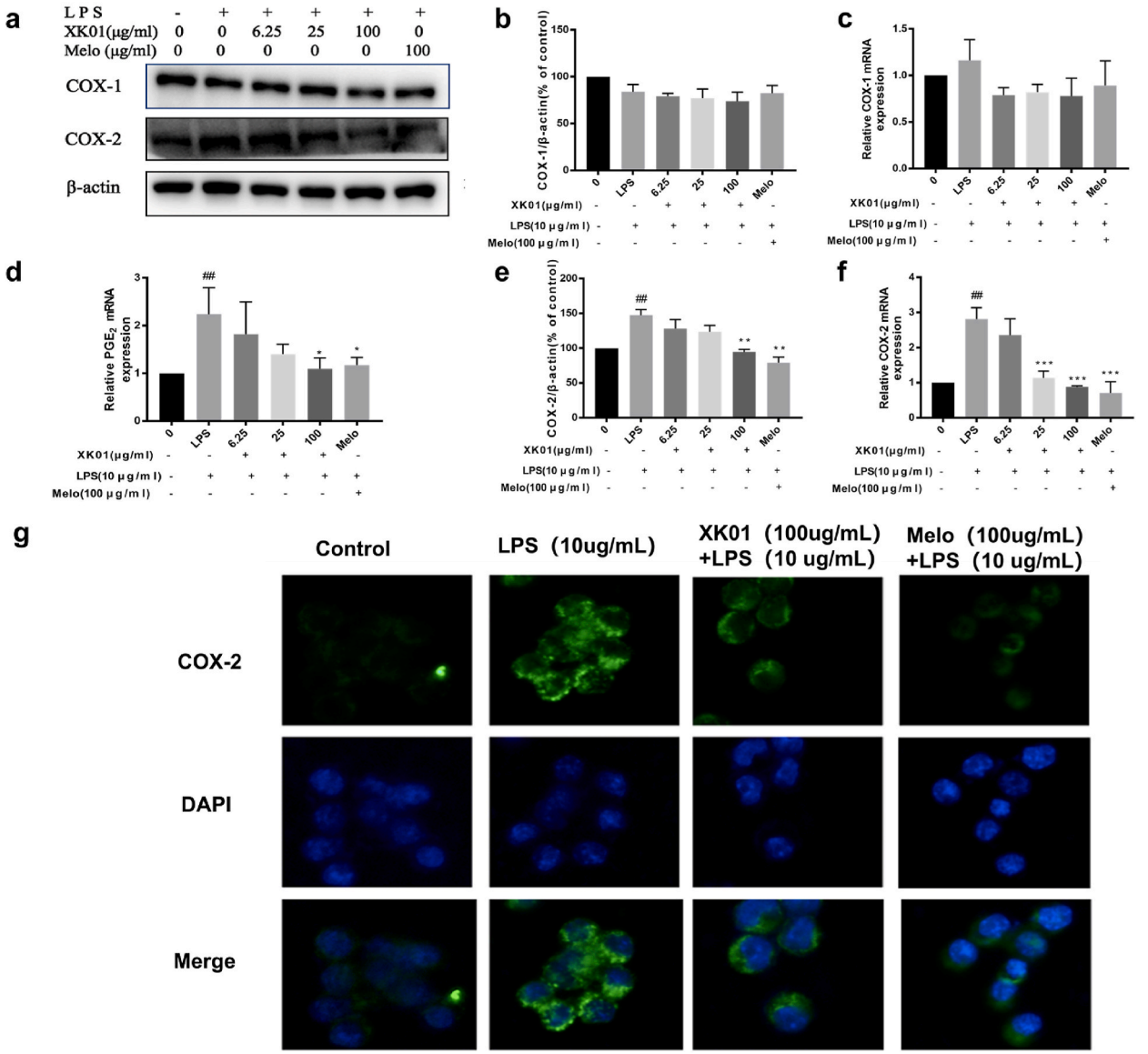

Fig. 5. XK01 inhibited the expression of COX-2 and PGE2 in LPS-activated RAW264.7 mouse macrophages. RAW 264.7 Cells were pretreated with Melo and different concentrations of XK01 followed by LPS stimulation. a: Western blot was used to detect the expression. of COX-1 and COX-2 proteins. b and e: Quantification of COX-1 and COX-2 proteins respectively. c, d, f: The mRNA expression of COX-1, PGE2 and COX-2 detected by real-time qPCR. g: COX-2 was detected by immunofluorescence assay. Graphs show mean ± SEM of at least three independent experiments. *P < 0.05. **P < 0.01, ***P < 0.001, $^{\#\#}$P < 0.01 from Student's t-test.

3.7. XK01 inhibited the inflammatory response of the ear swelling model mice

Compared with the left ear, the right ear of the mouse was observed to be red and swollen after applying xylene for 1 h, indicating that the ear swelling model was successfully established. The data results show that, compared with the model group, the Melo group and the XK01 low-dose, medium-dose and high-dose groups could reduce ear swelling. Compared with the meloxicam group, the swelling inhibition rate of the Melo group was 52.62 %, and the swelling inhibition rate of the XK01 high-dose group reached 53.39 %, indicating that each dose group of XK01 can inhibit the swelling of mouse ear-pieces caused by xylene, and inhibit the inflammatory response in a dose-dependent manner (see Table 3).

The slices of the ear tissue stained by H&E (Fig. 8A) showed that there were significantly more inflammatory cells infiltrated in the tissue of the model group. In the tissue of Melo group. A small amount of inflammatory cell infiltration can be seen, which inhibits the inflammatory response, and more fibroblasts proliferate, mainly spindle-shaped proliferation, which may be involved in the repair after inflammatory injury. Inflammatory cell infiltration was found in the dermis of ear slices of mice in low-dose and medium-dose

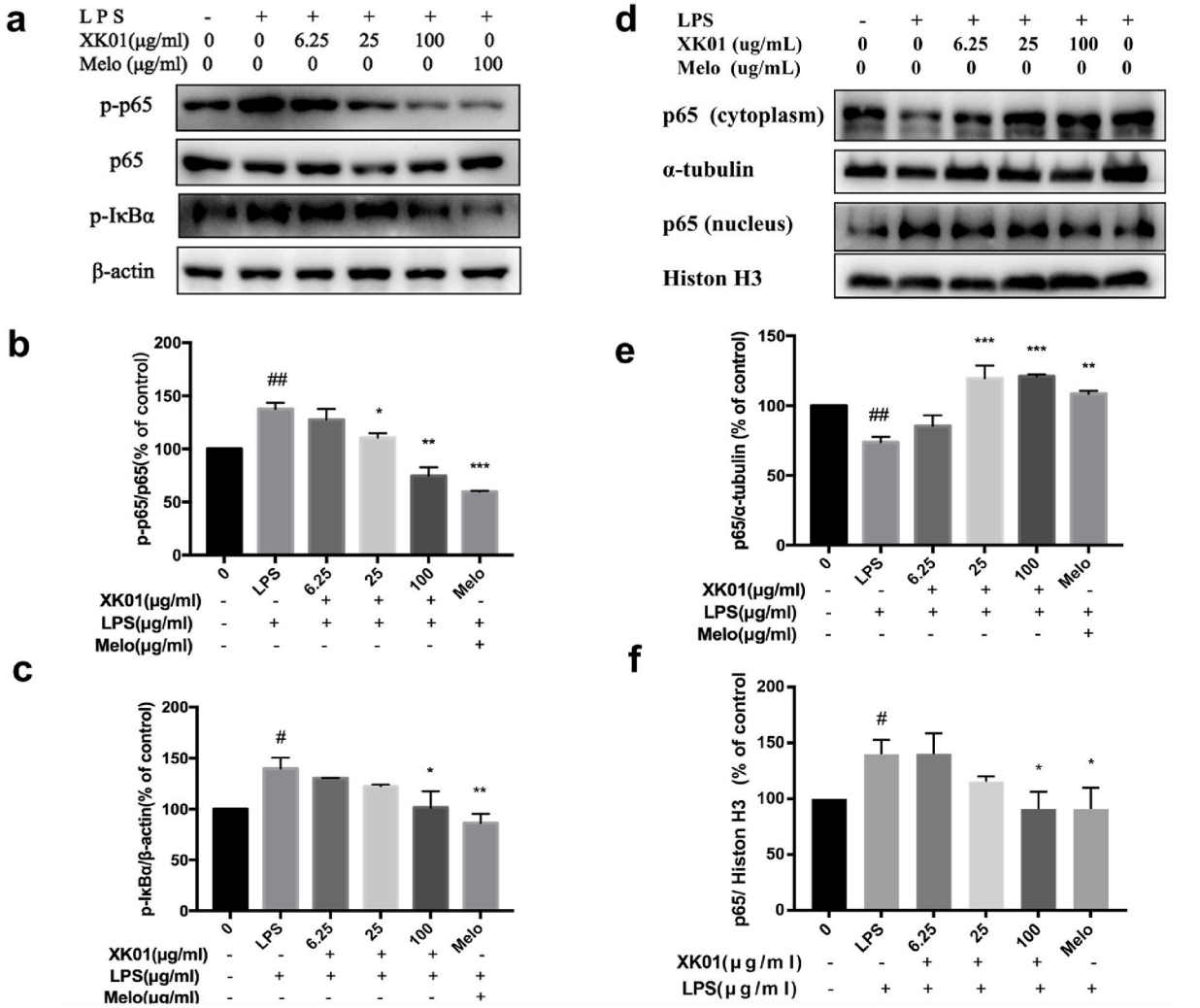

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Fig. 6. XK01 pretreatment inhibited NF-κB signaling in LPS-activated RAW264.7 mouse macrophages. RAW264.7 cells were pre-treated with different concentrations of XK01. a and d: Western blot was used to measure the expression of p-p65, p65, *p*-IκBα, nuclear and cytoplasmic p65; b, c, e and f: Quantification of p-p65/p65, *p*-IκBα/β-Action, p65/α-tubulin and p65/Histon H3; g: The effects of 100 μg/mL XK01 and Melo on NF-κB p65 nuclear translocation immunofluorescence assay. Graphs show mean ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 from Student's t-test.

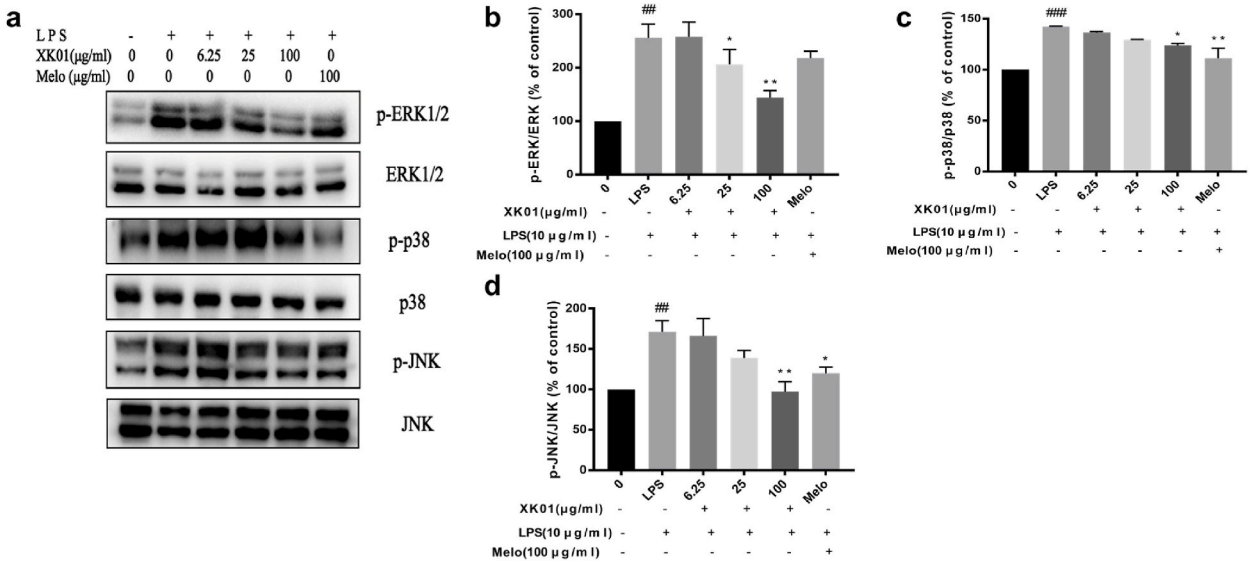

Fig. 7. XK01 inhibited the expression of phosphorylated proteins of ERK1/2, JNK and P38 in MAPK. LPS-stimulated RAW264.7 cells were pre-treated with different concentrations of XK01. a: Western blot was used to detect the expression of ERK1/2, *p*-ERK1/2, JNK, *p*-JNK, P38 and p-P38; b–d: Quantification of *p*-ERK1/2/ERK1/2, p-p38/p38 and *p*-JNK/JNK. Graphs show mean ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, $^{\#\#}$P < 0.01 from Student's t-test.

Table 3
XK01 inhibited the inflammatory response of the ear swelling model mice (n = 5, x ± s) (*P < 0.05, **P < 0.01 vs. Model).

Group	Concentration (mg/kg)	Ear swelling (mg)	Swelling inhibition rate(%)
Model	–	7.71 ± 0.80	–
Melo	6.0	3.65 ± 0.45**	52.62
XK01-LD	1.5	5.97 ± 0.95*	22.57
XK01-MD	3.0	4.72 ± 0.39*	38.74
XK01-HD	6.0	3.59 ± 0.51**	53.39

groups of XK01, while the infiltration of inflammatory cells in high-dose XK01 group was significantly improved. Moreover, We further assessed the expression of NF-κB in the ear swelling model mice by using IHC staining (Fig. 8B). Similarily, The expression of NF-κB is high in the tissue of the model group. Although the NF-κB expression in the low-dose roup didn't change, the medium-dose groups of XK01 and high-dose XK01 group inhibit the expression of NF-κB the same as Melo group. Therefore, from the histopathological studies, it can be shown that high-dose XK01 has a certain inhibitory effect on the xylene-induced inflammation model in mice via inhibiting NF-κB expression.

4. Discussion

The inflammatory response is a normal response of the body to eliminate harmful stimuli and is involved in host immune defence. When inflammation is poorly developed, it induces the release of intracellular inflammatory mediators and inflammatory factors, accelerating the development of inflammation and its complications, the most common of which are rheumatoid arthritis, colitis, sepsis and multiple sclerosis [17,18].

LPS is a TLR4 signalling activator that is highly active and triggers many inflammatory interlocking responses and macrophage activation upon entry into the organism [19]. Currently, the use of LPS to induce inflammation in macrophages is now widely accepted experimentally adopted as an in vitro model of cellular inflammation. Studies have shown that when RAW246.7 mouse monocyte macrophages are stimulated by LPS, the inflammatory response is activated, and a large number of inflammatory mediators and inflammatory factors such as NO, IL-1β, IL-6, TNF-α, etc., are synthesised and released from macrophages, which directly or indirectly

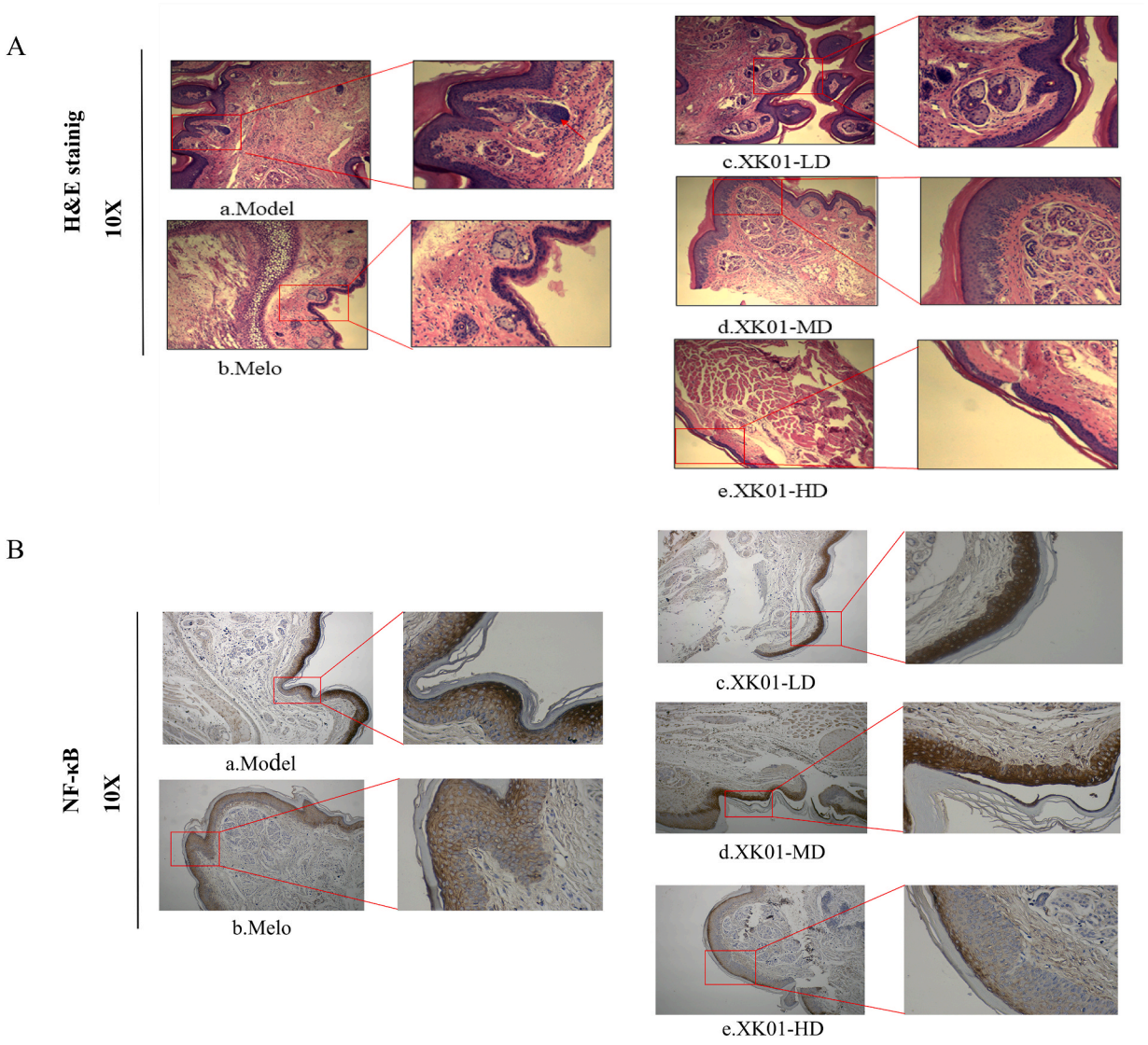

Fig. 8. XK01 inhibited the inflammatory response of the ear swelling model mice (10× and 40 ×). a: Histological assessments of organs with H&E staining in the tissue of ear swelling model mice treated with xylene, melo and different concentration of XK01. b: Representative IHC staining of NF-κB p65.

induces and activates other inflammatory cells to participate in the inflammatory response, exacerbating the worsening of inflammation, and leading to a more severe under the continuous worsening of the Organic damage [20].

In this study, we investigated the effects of a newly developed nonsteroidal XK01 on LPS-induced inflammation in vitro. Similar to meloxicam, XK01 with a concentration of 50 μg/mL or more was low cytotoxic to the mouse macrophages. ROS production induced by LPS can elicit a pro-inflammatory response by upregulating levels of the pro-inflammatory cytokines that act as messengers in subsequent processes, and it interacts with NO to generate more lethal intermediates, leading to cytotoxic damage [21]. Our data indicated that after pretreatment with different concentrations of XK01, IL-1β, IL-6, TNF-α, and NO levels were significantly decreased in a dose-dependent manner. And the present findings indicated that XK01 exerted potent scavenging effect on cellular ROS production.

COX is one of the most prominent targets of NSAIDs and promotes the formation of inflammatory and analgesic prostaglandins (PG) [22]. Non-steroidal anti-inflammatory drugs may achieve anti-inflammatory and analgesic pharmacological effects by inhibiting the activity and expression of cyclooxygenase.。XK01 can inhibit the COX-2 at protein and mRNA levels in a dose-dependent manner. At the same time, the expression of the COX-2 product prostaglandin is also reduced, and XK01 at 100 μg/mL showed similar effects as positive control meloxicam. In the present study, XK01 showed no significantly effects on COX-1, which indicates that the new compound XK01 may be a new NSAID of selective COX-2 inhibitor.

Nuclear factor NF-kB is an important transcription factor that controls the transcription of a variety of cellular genes such as COX-2, regulating the inflammatory response [23]. The new compound XK01 significantly inhibited the phosphorylation of NF-κB upstream

molecules, including IκBα and NF-κB p65. After translocation from the cytoplasm to the nucleus, they engage in transcriptional activation of target genes, various inflammatory factors are transcribed. A strong fluorescence intensity of NF-κB was detected in the nucleus in response to LPS exposure, which was blocked by XK01 pretreatment, indicating the XK01 prevented the entry of NF-κB into the nucleus. Therefore, the anti-inflammatory response of the new compound XK01 is regulated by NF-κB signaling pathway.

The MAPK signaling pathway, including ERK1/2, JNK and p38 MAPK, is widely involved in the regulation of cell life activities [24]. It well known that LPS activates the phosphorylated expression of p38, ERK and JNK in RAW264.7. Pretreatment with XK01 attenuated the activation of the indicated members of MAPK family. In this process, the effect of XK01 on *p*-ERK1/2 and *p*-JNK are more potential that on p-p38, and XK01 exerted a stronger effect when compared to the same dose of meloxicam [25].

In this study, we explored that different concentrations of XK01 could reduce the concentration of NO and scavenge ROS. XK01 could also inhibit the phosphorylation of p65 and IκB and MAPKs proteins, block the activation of NF-κB, MAPK pathway, and inhibit the release of inflammatory mediators, such as COX-2, which leads to the conclusion that XK01 has the potential to become a novel anti-inflammatory drug. However, whether XK01 down-regulates the expression and activity of COX enzymes via JNK and ERK in the MAPK pathway and what roles it plays in JNK, ERK and MAPK pathways need to be further investigated; and we also need to optimize the synthetic route and technology of XK01, aiming to obtain a new meroxicam non-steroidal compound that is more effective and has a better safety profile.

5. Conclusions

XK01 can reduce NO level, scavenge ROS, and down-regulated the levels of COX-2, TNF-α, IL-6, and IL-1β, which suggests that XK01 may be a potential anti-inflammatory agent. Regarding the molecular mechanisms, XK01 alleviates the inflammatory response via inhibition of the release of COX-2 and other inflammatory mediators, which was at least partially by down-regulating the activation of MAPK and NF-κB signaling pathways induced by LPS in RAW264.7 cells.

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Ethics approval

The animal study protocol was approved by the Biomedical Research Ethics Committee of Hunan Normal University (D2021068).

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Jixiang Wang: Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Jiawang Tan:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Qianmei Hu:** Writing – original draft, Validation, Methodology, Conceptualization. **Siyu Mao:** Writing – original draft, Validation, Investigation. **Hongting Chen:** Validation, Investigation, Formal analysis. **Weiyi Luo:** Validation, Investigation. **Xing Feng:** Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.\

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24004.

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