Original Communication

Capsaicin affects macrophage anti-inflammatory activity via the MAPK and NF-κB signaling pathways

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Abstract: Capsaicin, the main constituent in chili, is an extremely spicy vanillin alkaloid and is found in several *Capsicum* species in China. Traditionally, it has been used to treat inflammatory diseases such as allergic rhinitis, neuralgia after shingles, refractory female urethral syndrome, spontaneous recalcitrant anal pruritus, and solid tumors. Constant stimulation of the body by inflammatory factors can lead to chronic inflammation. Capsaicin possesses anti-inflammatory activity; however, the underlying mechanism is unknown. We investigated the effect of capsaicin on the secretion of macrophage inflammatory factors in a lipopolysaccharide-induced inflammation model using 56 healthy, SPF grade, BALB/c mice. To this end, mice peritoneal macrophages were isolated and stimulated with lipopolysaccharide (1 μg/mL) and capsaicin (25, 50, 75, or 100 μg/mL) for 24 h. At all concentrations tested, capsaicin significantly promoted the phagocytosis of neutral red dye by macrophages. Furthermore, the gene expression and secretion of inflammatory cytokines significantly increased after induction with lipopolysaccharide ($P<0.01$); the interleukin (IL)-6 level was 204 μg/mL, tumor necrosis factor (TNF)-α level was 860 μg/mL, and nitric oxide (NO) level was 19.8 μg/mL. However, the treatment with capsaicin reduced their levels ($P<0.01$) and protein expression of lipopolysaccharide-induced extracellular signal-related kinase 1/2 and p65 ($P<0.05$). Overall, capsaicin reduced the secretion of inflammatory cytokines ($P<0.01$), interleukins, TNF-α ($P<0.01$), and NO by inhibiting the nuclear factor-kappa B and microtubule-associated protein kinase signaling pathways, and thereby reduced lipopolysaccharide-induced inflammatory response in macrophages.

Keywords: capsaicin, inflammation, cytokine, signaling pathway

Abbreviations

ACTB	Beta-actin
AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCL-2	Chemokine ligand 2
Cox-2	Cyclooxygenase-2
DHVA	N-docosahexaenoylvanillylamine
ELISA	Enzyme-linked immunosorbent assay
EPVA	N-eicosapentaenoyl vanillylamine
ERK1/2	Extracellular signal-related kinase 1/2
FFA	Free fatty acid
HRP	Horseradish peroxidase
iNOS	Inducible nitric oxide synthase
IL-6	Interleukin-6
LPS	Lipopolysaccharide
LSD	Least significant difference
MAPK	Microtubule associated protein kinase
McP-1	Monocyte chemoattractant protein I
MHC-II	Major histocompatibility complex II
MIP-1	Macrophage inflammatory protein-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator activated receptor
PVDF	Polyvinylidene difluoride
RPMI	Rosewell Park Memorial Institute
SD	Standard deviation
THP-1	Human acute monocytic leukemia cell line
TNF-α	Tumor necrosis factor-alpha
TRPV	Transient receptor potential cation channel subfamily V member 1

Introduction

Inflammation is a pathophysiological phenomenon associated with several diseases and a defensive immune response to vascular trauma. During inflammation, acute inflammatory cells and macrophages are activated, resulting in the release of a series of pro-inflammatory cytokines and mediators, including nitric oxide (NO), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 [1, 2]. These pro-inflammatory factors play a role in dilating blood vessels and stimulating chemotactic responses to inflammation [3]. Steroids and non-steroidal drugs are widely used anti-inflammatory drugs, but they are associated with serious adverse effects on the gastrointestinal tract, kidneys, and central nervous system [4]. Therefore, it is imperative to develop anti-inflammatory drugs with improved efficiency and low adverse effects to treat chronic inflammation.

Capsaicin is an important bioactive alkaloid isolated from *Capsicum* species (Figure 1). It has shown promising effects in cancer prevention, pain relief, and weight loss [5]. Currently, capsaicin is used as an anticancer agent [6], an antioxidant [7], a food additive, a diet supplement, a topical analgesic, and an antipruritic medication [8].

Studies have reported that after the induction of inflammatory signals, two main intracellular signaling pathways regulate inflammatory response in macrophages. Mitogen-activated protein kinase (MAPK) and transcription nuclear factor (NF)-β are the main components of the signal transduction pathways [8]. MAPK belongs to the serine/threonine protein kinase family and consists of three major subunits, namely, erk1/2, jnk1/2, and p38. All three MAPK subunits can be activated by various extracellular stimuli in macrophages, such as LPS, cytokines, and polysaccharides [9]. NF-β is a key transcription factor of several genes that regulate immune and inflammatory responses. It is an important nuclear transcription factor and a fast-response cytoplasmic transcription factor. NF-β is located at the junction of the downstream TLR signaling pathways and is involved in immune response and cell proliferation and differentiation. It plays an important regulatory role in cell growth, differentiation, adhesion, apoptosis, and inflammatory response [10]. However, information on the effect of capsaicin on the MAPK and NF-β signaling pathways in macrophages is limited.

Capsaicin improved the inflammatory response induced by free fatty acids by reducing the expression of macrophage inflammatory protein-1 (MIP-1) and interleukin 8 (IL-8) in macrophages and human acute mononuclear leukemia THP-1 cells [11]. It attenuated palmitate-induced expression of MIP-1 and IL-8 by increasing palmitate oxidation and reducing c-Jun activation in THP-1 cells [12]. Furthermore, capsaicin inhibited the production of TNF-α in 264.7 macrophages by activating peroxisome proliferator-activated receptor (PPAR)

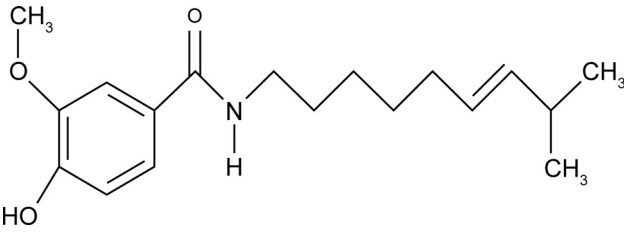

Figure 1. Chemical structure of capsaicin.

[11]. Capsaicin also regulated the expression of cyclooxygenase-2 (Cox-2) and induced nitric oxide synthase (iNOS) and vanilloid receptor-1 (Vr-1) in lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages, probably via a novel mechanism rather than the Vr-1 receptor [12].

Obesity-induced inflammatory response can be reduced by inhibiting the release of adipokines from adipose tissues in obese mice and the increase in the number of macrophages. Capsaicin has been shown to inhibit the expression of IL-6 and monocyte chemoattractant protein (McP-1) in the adipose tissues of obese mice; therefore, it can be used to reduce inflammation caused by obesity and its related complications [13]. Capsaicin has also been shown to activate transient receptor potential cation channel subfamily V member 1 (TRPV 1) and inhibit fat formation, thereby enabling weight loss [14, 15]. The capsaicin analogs N-eicosapentaenoyl vanillylamine (EPVA) and N-docosahexaenoyl vanillylamine (DHVA) can significantly inhibit the production of 264.7 macrophages and inflammatory mediators such as NO, macrophage inflammatory protein-3 prime, and McP-1 (or CCL2), induced by LPS [16].

However, there are only a few studies on the anti-inflammatory activity and action mechanism of capsaicin. Therefore, in the present study, we evaluated the effects of capsaicin on the secretion of inflammatory factors and the underlying mechanism using a cellular inflammatory model established using LPS-induced mouse peritoneal macrophages. Our study provides an experimental basis for the clinical application of capsaicin to treat inflammation.

Materials and methods

Mice and materials

BALB/c mice were provided by Jinzhou Medical University. Capsaicin was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Rosewell Park Memorial Institute 1640 (RPMI-1640) medium and fetal bovine serum were obtained from Gibco (Beijing, China). 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) and LPS were purchased from Sigma (St. Louis, MO, USA). IL-6, TNF-α, and mouse NO enzyme-linked immunosorbent assay (ELISA) kits were bought from Shanghai Lanpai Biotechnology Co., Ltd. (Shanghai, China), Wuhan Chundu Biological Technology Co., Ltd. (Shanghai, China), and Shanghai Bangyi Biotechnology Co., Ltd. (Shanghai, China), respectively. TaKaRa RNA PCR Kit for Avian Myeloblastosis Virus ver. 3.0 was obtained from Bao Biological Engineering Co., Ltd. (Dalian, China). Bovine serum albumin was purchased from Amresco (Shanghai, China). Rabbit monoclonal anti-β-actin (42 kD) and horseradish peroxidase (HRP)-sheep anti-rabbit secondary antibodies were acquired from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Primary antibodies against ERK (42 kD), p-ERK (44 kD), NF-κB-p65 (65 kD), and p-NF-κB-p65 (65 kD) were purchased from Cell Signaling Technology (Wuhan, China). Under aseptic conditions, LPS solution, RPMI-1640 complete medium, dexamethasone solution, and capsaicin solution of different concentrations and other reagents were prepared and stored at 4 °C until further use. Bacterial suspension was passed through microporous filtration membrane, and the filtrate was stored at 4 °C.

All animal experiments were approved by the Ethics Committee and the Experimental Animal Center of Jinzhou Medical University (ethics approval number 2018008) and were performed in accordance with the local ethical guidelines.

Isolation and culture of macrophages

The mice were sacrificed via cervical dislocation to isolate macrophages. For this purpose, the skin on the abdomen of mice was sterilized with iodine and then with 75% ethanol. The peritoneal cavity was injected with 5 mL of precooled RPMI-1640 culture medium, and the abdomen was rubbed for 2–3 min. The peritoneal fluid was aspirated and collected into a centrifuge tube; the process was repeated twice. The cell suspension was centrifuged (Hexi Centrifuge Co., Ltd., Changsha, Hunan, China) at $110\times g$ for 10 min, and macrophages were collected from the cell pellet. Live cells were counted by trypan blue staining, seeded in 96-well cell culture plates, and incubated at 37 °C under 5% CO2 for 2 h. Thereafter, the cells that adhered to the culture plates were purified and counted as mouse peritoneal macrophages.

Effect of capsaicin on the phagocytic activity of peritoneal macrophages

The following four groups were tested: the blank, LPS, dexamethasone, and capsaicin treatment groups. Briefly, 100 μL of macrophage suspension was added into wells of a 96-well culture plate. Each treatment group was tested in duplicate. For each group, 100 μL of the corresponding solution was added to each well (the blank group, RPMI-1640 complete medium; the LPS group, LPS solution 1 μg/mL; the dexamethasone group, dexamethasone solution 10 μg/mL; and the capsaicin group, capsaicin solution of different concentrations: 25, 50, 75, and 100 μg/mL). The macrophages were treated for 30 min before discarding the supernatant. The cells were washed with phosphate buffered saline (PBS) and then incubated for 30 min with 1 g/L neutral red solution per well. The supernatant was discarded, and the cells were washed with PBS three times and lysed with 100 μL of cell lysis solution per well at 4 °C for 12 h. The absorbance of the sample at 570 nm was measured using a plate reader (Sunrise-Basic, Tecan, Austria).

Effects of capsaicin on IL-6, TNF-α, and NO secretion by peritoneal macrophages

Lipopolysaccharides (1 μg/mL), dexamethasone (10 μg/mL), and capsaicin (25, 50, 75, and 100 μg/mL) were added to peritoneal macrophage cultures, which were incubated for 24 h; then, inflammatory mediators were purified. Peritoneal macrophages (200 μL containing 1.0×10^5 cells) were plated in each well of 96-well microplates. The cells were treated with capsaicin (25, 50, 75, or 100 μg/mL) and cultured for 48 h. The IL-6, TNF-α, and NO levels in the cell culture supernatants were determined using the ELISA kits, according to the manufacturer's guidelines (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). The absorbance of the solution was measured at 450 nm using an enzymatic plate reader (Sunrise-Basic; Tecan, Austria). The levels of IL-6, TNF-α, and NO in the supernatant were determined using the mouse IL-6, TNF-α, and NO biotin double antibody sandwich ELISA kits (Solarbio Science and Technology Co., Ltd.). Five replicates were set for each concentration of the standard substance and sample, and concentration zero was set for the blank well. The absorbance (optical density) of the sample in each well was measured at 450 nm using a microplate analyzer, and standard curves were drawn separately to calculate the levels of IL-6, TNF-α, and NO in all samples.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Peritoneal macrophages were seeded in a 24-well plate at a density of 5×10^6 cells/mL. The following four groups were tested: the blank, LPS (final concentration 1 μg/mL), dexamethasone (final concentration 10 μg/mL), and capsaicin treatment groups (final concentration 25, 50, 75, and 100 μg/mL) for 24 h. The total RNA was extracted from

peritoneal macrophages and used to synthesize the first complementary DNA (cDNA) strand using the PrimerScript RT reagent kit (Takara, Dalian, China), according to the manufacturer's instructions. Specific primers for IL-6, TNF-α, iNOS, and ACTB (housekeeping gene) were designed using Primer Premier 5.0 software (Premier Company, USA) and synthesized by Shanghai Biotechnology Co., Ltd. (Shanghai, China). The primer sequences used are listed in Table E1 in the electronic supplementary material (ESM) 1. The polymerase chain reaction (PCR) conditions were as follows: denaturation at 94 °C for 5 min, 36 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. The PCR products were identified by gel electrophoresis on 1.5% agarose, as previously described [9].

Effect of capsaicin on the phosphorylation of MAPK subunit ERK1/2 and NF-κBp65

Mice peritoneal macrophages were seeded in 24-well plates at a concentration of 2×10^6/well. The following four different groups were tested: the blank (RPMI-1640 complete medium), LPS (final concentration 1 μg/mL), dexamethasone (final concentration 10 μg/mL), and capsaicin treatment groups (final concentration 25, 50, 75, or 100 μg/mL).

The cells were cultured at 37 °C under 5% CO_2 for 24 h, collected, and centrifuged for 10 min at 132×g at 4 °C. Total proteins were purified, and their concentrations were determined using the bicinchoninic acid (BCA) method according to the manufacturer's guidelines (Solarbio Science and Technology Co., Ltd.). The phosphorylation of MAPK subunit ERK1/2 and NF-κBp65 was analyzed by western blotting. Briefly, the proteins were separated by electrophoresis on a 15% polyacrylamide gel and transferred on to polyvinylidene difluoride (PVDF) membrane using the wet transfer method. The membrane was incubated with the primary antibody for 12 h (rabbit monoclonal antibody, 1:1000) and secondary antibody (HRP-sheep anti-rabbit, 1:1000) for 1 h. The blots were exposed to X-ray film, and the protein bands were detected. The experiment was repeated three times, and the protein band intensities were calculated and normalized to that of β-actin using Image J software (National Institutes of Health, USA).

Statistical analysis

All experiments were repeated at least three times independently, and all data are presented as mean±standard deviation (STDEV). SPSS 20.0 (SPSS, Chicago, IL, USA) software was used to perform a one-way analysis of variance (one-way ANOVA). The LSD method was used for pairwise comparison between groups. Results with $P<0.05$ were considered significantly different.

Results

Effects of capsaicin on the phagocytic function of macrophages

As shown in Table E2 in ESM 1, the phagocytic activity of peritoneal macrophages in the capsaicin, LPS, and dexamethasone groups was higher than that in the blank group ($P<0.05$).The phagocytic activity of peritoneal macrophages increased with the concentration of capsaicin only in the range of 25–100 μg/mL. The phagocytic activity of peritoneal macrophages in the 50 and 75 μg/mL capsaicin groups was significantly higher than that in the LPS group ($P<0.01$), but not in the 25 and 100 μg/mL capsaicin groups ($P>0.05$).

The phagocytic activity of peritoneal macrophages in the capsaicin treatment groups was higher than that in the LPS group but lower than that in the dexamethasone group. The phagocytic activity of peritoneal macrophages in all capsaicin treatment groups, except the 50 μg/mL capsaicin treatment group, was significantly lower than that in the dexamethasone group ($P<0.05$).

Effects of capsaicin on the secretion of IL-6, TNF-α, and NO by mouse peritoneal macrophages

The secretion of IL-6, TNF-α, and NO in the capsaicin treatment groups was higher than that in the blank control group ($P<0.01$). The secretion of IL-6, TNF-α, and NO in the capsaicin treatment groups was lower than that in the LPS group ($P<0.01$). The secretion of IL-6, TNF-α, and NO in all capsaicin treatment groups was higher than that in the dexamethasone group ($P<0.01$), as shown in Table 1.

To determine the effect of capsaicin on LPS-induced inflammation, we treated macrophages with LPS and capsaicin. Peritoneal macrophages were stimulated with LPS (1 μg/mL) and capsaicin (final concentration: 25, 50, 75, and 100 μg/mL) for 24 h. Capsaicin blocked LPS-induced secretion of IL-6, TNF-α, and NO in peritoneal macrophages, and the inhibitory effect increased with capsaicin concentration. The secretion of IL-6 and TNF-α in the LPS+ capsaicin groups was higher than that in the blank control group ($P<0.01$). The levels of IL-6, TNF-α, and NO in the LPS+ capsaicin group were lower than those in the LPS group ($P<0.01$). The levels of IL-6 and TNF-α in the capsaicin group were higher than those in the dexamethasone group ($P<0.01$). NO secretion in the LPS+25 μg/mL

Table 1. Effect of capsaicin on the secretion of IL-6, TNF-α, NO by macrophages

Group	IL-6 (μg/mL)	TNF-α (μg/mL)	NO (μg/mL)
Blank	121±9.69^{Gh}	666±61.3^{Gg}	29.2±0.03^{Dd}
LPS	436±0.76^{Aa}	2,024±13.2^{Aa}	52.4±2.15^{Aa}
Dexamethasone	203±3.39^{Ff}	1,012±10.8 ^{Ee}	34.0±0.53^{Cc}
25 μg/mL capsaicin	388±15.35^{Bb}	1,510±11.9^{Bb}	38.6±1.79^{Bb}
50 μg/mL capsaicin	287±20.89^{Dd}	1,506±70.7^{Bb}	38.1±0.55^{BCbc}
75 μg/mL capsaicin	251±9.64^{Ee}	1,205±88.6^{Dd}	35.4±2.42^{BCc}
100 μg/mL capsaicin	215±4.09^{Ff}	1,014±68.2^{Ee}	35.1±0.72^{BCc}
LPS+25 μg/mL capsaicin group	361±3.20^{Cc}	1,333±46.0^{Cc}	38.6±2.66^{Bb}
LPS+50 μg/mL capsaicin	258±2.07^{Ee}	1,318±40.7^{CDc}	30.41±2.12^{CDd}
LPS+75 μg/mL capsaicin	219±2.02^{Ff}	1,057±16.0^{Ee}	21.80±3.39^{Ee}
LPS+100 μg/mL capsaicin	204±7.89^{Ff}	860±37.3^{Ff}	19.89±1.14^{Ee}

Compared with the blank group, different lowercase letters in the same column indicate a significant difference (P<0.05), whereas the same indicate no significant difference (P>0.05). The uppercase letters in the same column indicate a very significant difference (P<0.01). IL-6: interleukin 6; TNF-α: tumor necrosis factor-α; NO: nitric oxide; LPS: lipopolysaccharide.

Table 2. Effect of capsaicin on the expression of cytokine IL-6, TNF-α, NO mRNA secreted by mouse peritoneal macrophages

Group	IL-6 (μg/mL)	TNF-α (μg/mL)	NO (μg/mL)
Blank	121±9.69^{Gh}	666±61.3^{Gg}	29.2±0.03^{Dd}
LPS	436±0.76^{Aa}	2,024±13.2^{Aa}	52.4±2.15^{Aa}
Dexamethasone	203±3.39^{Ff}	1,012±10.8 ^{Ee}	34.0±0.53^{Cc}
25 μg/mL capsaicin	388±15.35^{Bb}	1,510±11.9^{Bb}	38.6±1.79^{Bb}
50 μg/mL capsaicin	287±20.89^{Dd}	1,506±70.7^{Bb}	38.1±0.55^{BCbc}
75 μg/mL capsaicin	251±9.64^{Ee}	1,205±88.6^{Dd}	35.4±2.42^{BCc}
100 μg/mL capsaicin	215±4.09^{Ff}	1,014±68.2^{Ee}	35.1±0.72^{BCc}
LPS+25 μg/mL capsaicin group	361±3.20^{Cc}	1,333±46.0^{Cc}	38.6±2.66^{Bb}
LPS+50 μg/mL capsaicin	258±2.07^{Ee}	1,318±40.7^{CDc}	30.41±2.12^{CDd}
LPS+75 μg/mL capsaicin	219±2.02^{Ff}	1,057±16.0^{Ee}	21.80±3.39^{Ee}
LPS+100 μg/mL capsaicin	204±7.89^{Ff}	860±37.3^{Ff}	19.89±1.14^{Ee}

Compared with the blank group, different lowercase letters in the same column indicate a significant difference (P<0.05), whereas the same indicate no significant difference. The uppercase letters in the same column indicate a very significant difference (P<0.01). iNOS: inducible nitric oxide synthase.

capsaicin group was higher than that in the dexamethasone group (P>0.05). NO secretion in the LPS+50, LPS+75, and LPS+100 μg/mL capsaicin groups was also lower than that in the dexamethasone group (P>0.05). However, NO secretion in the LPS+25 μg/mL capsaicin group was higher than that in the dexamethasone group, as shown in Table 1.

Effects of capsaicin on macrophage-derived IL-6, TNF-α, and iNOS gene expression

Peritoneal macrophages were treated with LPS (1 μg/mL), dexamethasone (10 μg/mL), and capsaicin (25, 50, 75, and 100 μg/mL) for 24 h. The mRNA levels of *IL-6*, *TNF-α*, and *iNOS* in the capsaicin groups were lower than those in the blank group (P<0.01). The mRNA levels of *IL-6*, *TNF-α*, and *iNOS* in the capsaicin groups were lower than those in the LPS group (P<0.01). However, the mRNA levels of *IL-6*, *TNF-α*, and *iNOS* in the capsaicin groups (at all doses) were higher than those in the dexamethasone group (P<0.01), as shown in Table 2 and Figure 2A.

To investigate whether capsaicin could alleviate LPS-induced inflammation, peritoneal macrophages were stimulated with LPS (1 μg/mL) and capsaicin (final concentrations 25, 50, 75, and 100 μg/mL) simultaneously for 24 h. The mRNA expression of IL-6, TNF-α, and *iNOS* in the LPS + capsaicin groups was lower than that in the blank group (P<0.01). The *IL-6*, *TNF-α*, and *iNOS* mRNA levels were lower in the LPS+ capsaicin groups than in the LPS group and were significantly higher than those in the dexamethasone group (P<0.01), as shown in Table 2 and Figure 2B.

Effects of capsaicin on the expression of ERK protein and its phosphorylated form

Peritoneal macrophages were stimulated with LPS (1 μg/mL) or dexamethasone (10 μg/mL) in combination with capsaicin (25, 50, 75, or 100 μg/mL) for 24 h. The expression of ERK in the LPS+capsaicin groups was higher than

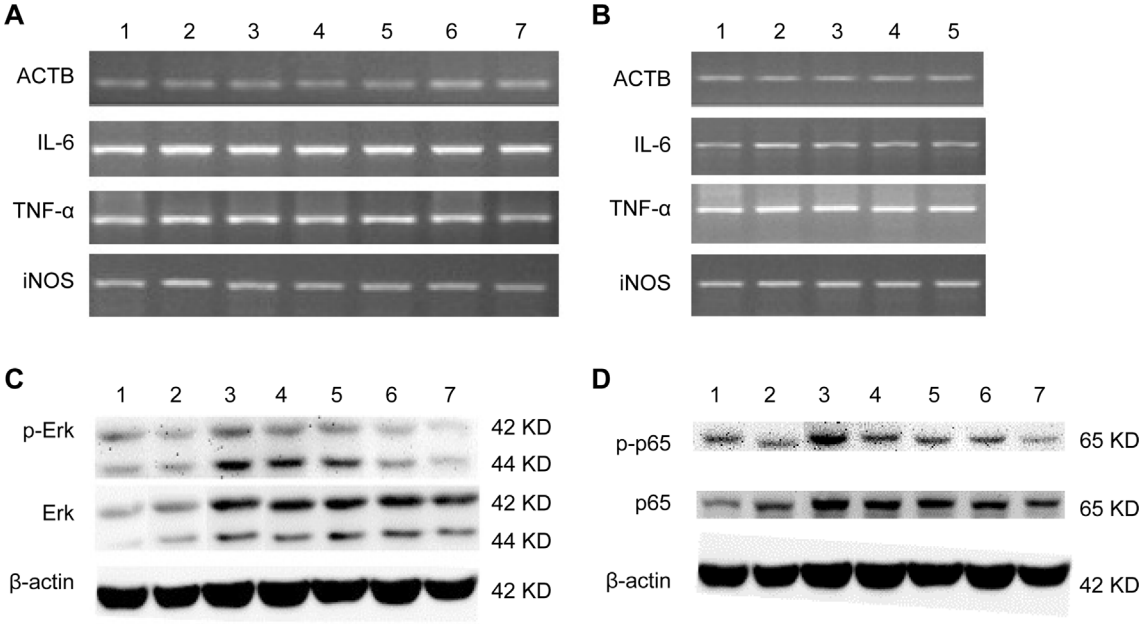

Figure 2. A) IL-6, TNF-α, and iNOS mRNA levels (normalized to ACTB). 1. Blank, 2. LPS, 3. 25 μg/mL capsaicin, 4. 50 μg/mL capsaicin, 5. 75 μg/mL capsaicin, 6. 100 μg/mL capsaicin, and 7. dexamethasone; B) IL-6, TNF-α, and iNOS mRNA levels (normalized to ACTB). 1. LPS, 2. LPS+25 μg/mL capsaicin, 3. LPS+50 μg/mL capsaicin, 4. LPS+75 μg/mL capsaicin, and 5. LPS+100 μg/mL capsaicin; C) Expression of ERK and p-ERK in peritoneal macrophages. 1. Blank, 2. LPS+dexamethasone, 3. LPS, 4. LPS+25 μg/mL capsaicin, 5. LPS+50 μg/mL capsaicin, 6. LPS+75 μg/mL capsaicin, and 7. LPS+100 μg/mL capsaicin; D) Expression of p65 and p-p65 in peritoneal macrophages. 1. Blank, 2. LPS+dexamethasone, 3. LPS, 4. LPS+25 μg/mL capsaicin, 5. LPS+50 μg/mL capsaicin, 6. LPS+75 μg/mL capsaicin, and 7. LPS+100 μg/mL capsaicin.

that in the blank group (P<0.01). The phosphorylation level of ERK was higher in the LPS+25 and LPS+50 μg/mL capsaicin groups, but lower in the LPS+75 and LPS+100 μg/mL capsaicin groups, than in the blank group (for both comparisons, P<0.01) (Figures E1–E3 in ESM 1).

The expression of ERK and its phosphorylated form in the LPS+capsaicin groups was lower than that in the LPS group (P<0.01). However, the levels of both proteins increased in the LPS+25 and LPS+50 μg/mL capsaicin groups, but decreased in the LPS+75 and LPS+100 μg/mL capsaicin groups (P<0.01) compared with those in the dexamethasone group, as shown in Figure 2C and Table 3.

The protein level of NF-κBp65 in the LPS+capsaicin groups was higher than that in the blank group (P<0.01). In addition, the phosphorylation level of NF-κB p65 was higher in the LPS+25 and LPS+50 μg/mL capsaicin groups but lower in the LPS+75 and LPS+100 μg/mL capsaicin groups than in the blank group (P<0.01) (Figures E4–E5 in ESM 1).

The expression of NF-κBp65 and its phosphorylated form in the LPS+capsaicin groups (at all concentrations) was lower than that in the LPS group (P<0.01 and P<0.05, respectively). The phosphorylation level of NF-κBp65 decreased in the LPS+50, LPS+75, and LPS+100 μg/mL capsaicin groups but increased in the LPS+25 μg/mL capsaicin group (P<0.01) compared with that in the dexamethasone group (Figure 2D and Table 3).

Discussion

Phagocytosis is a highly regulated function of macrophages, as basal phagocytosis by stationary and circulating macrophages is typically low. However, exposure to signals, such as antigens and cytokines, can activate macrophages and enhance their phagocytic functions. Xia showed that exposure to alkaloids improved the phagocytic function of mouse peritoneal macrophages using the MTT and neutral red assays [11]. In this study, the phagocytic ability of peritoneal macrophages was determined using the neutral red phagocytosis test; dexamethasone was used as the positive control. The results showed that capsaicin and dexamethasone significantly increased the phagocytic activity of mouse peritoneal macrophages *in vitro*.

Macrophages play an important role in inflammatory responses, and the cytokines they secrete, such as TNF-α, IL-1, and IL-6, play a key role in inflammation, tumors, and autoimmune diseases [11–13]. Therefore, the level of inflammatory cytokines secreted by macrophages can be used as an indicator of the anti-inflammatory effect of drugs. In this study, LPS-induced mouse peritoneal macrophages were used as a cellular inflammatory model to study the effect of capsaicin on the secretion of cytokines by macrophages and the underlying molecular mechanism. The levels of IL-6, TNF-α, and NO in the capsaicin groups were higher than those in the dexamethasone

Table 3. Effects of capsaicin on MAPK subunits ERK42/44, NF-κB, p65 protein, and their phosphorylated forms

Group	ERK/β-actin	p-ERK/β-actin	p65/β-actin	p-p65/β-actin
Blank	0.08 ± 0.003^{Cc}	0.16 ± 0.04^{CDc}	0.10 ± 0.01^{Dd}	0.07 ± 0.01^{Cc}
LPS	0.43 ± 0.033^{Aa}	0.40 ± 0.04^{Aa}	0.37 ± 0.04^{Aa}	0.35 ± 0.02^{Aa}
Dexamethasone	0.14 ± 0.017^{Cd}	0.10 ± 0.02^{Dd}	0.13 ± 0.03^{Dd}	0.07 ± 0.01^{Cc}
LPS+25 μg/mL capsaicin	0.38 ± 0.02^{Ab}	0.30 ± 0.03^{Bb}	0.28 ± 0.02^{Bb}	0.11 ± 0.03^{Bb}
LPS+50 μg/mL capsaicin	0.44 ± 0.03^{Aa}	0.19 ± 0.03^{Cc}	0.27 ± 0.04^{BCb}	0.07 ± 0.01^{Cc}
LPS+75 μg/mL capsaicin	0.42 ± 0.03^{Aab}	0.07 ± 0.01^{Dd}	0.21 ± 0.03^{Cc}	0.06 ± 0.006^{Cc}
LPS+100 μg/mL capsaicin	0.30 ± 0.05^{Bc}	0.05 ± 0.01^{De}	0.13 ± 0.03^{Dd}	0.04 ± 0.003^{Cc}

Compared with the blank group, different lowercase letters in the same column indicate a significant difference ($P<0.05$), whereas the same indicate no significant difference. The uppercase letters in the same column indicate a very significant difference ($P<0.01$). MAPK: microtubule associated protein kinase; ERK1/2: extracellular signal-related kinase 1/2; NF-κB: nuclear factor-kappa B.

groups, and this may be due to the low concentration of dexamethasone.

TNF-α and IL-1 are the most important inflammatory factors in the early stages of inflammation. These cytokines activate endothelial cells and act as chemotactic neutrophils to initiate the inflammatory mediator cascade reaction [14]. These early inflammatory factors can further induce the release of "secondary inflammatory factors," such as IL-6, IL-8, and NO [13]. Moreover, macrophage nonspecific immunity is closely related to the production of NO, an inflammatory mediator capable of killing target cells and exerting cytotoxicity against phagocytic tumor cells and microorganisms. It is also a carrier that can help exchange information between cells [15]. In our study, 24 h of incubation with 25, 50, 75, or 100 μg/mL capsaicin significantly reduced the mRNA and protein expression of IL-6, TNF-α, and iNOS in the LPS (1 μg/mL)-induced inflammation model of macrophages. The results suggested that capsaicin could not only inhibit the inflammatory factors in the early stages of inflammation but also further inhibit the inflammatory cascade reaction and reduce the release of "secondary inflammatory factors," and thus exert an anti-inflammatory effect against LPS-induced inflammation.

The results showed that the addition of 25, 50, 75, and 100 μg/mL capsaicin significantly inhibited the mRNA and protein expression of IL-6, TNF-α, and NO, and the phosphorylation of NF-κB p65 signaling molecules induced by LPS (1 μg/mL) in mouse peritoneal macrophages at 24 h. By inhibiting inflammatory cytokines IL-6, TNF-α, and iNOS, capsaicin inhibits the activation of LPS-induced NF-β pathway in macrophages, leading to decreased expression of inflammatory cytokines. This is one of the important molecular mechanisms underlying the anti-inflammatory effect of capsaicin.

NF-κB, usually referred to as the heterodimer of p50/p65, is involved in the transcriptional regulation of various cytokines and inflammatory mediators [16, 17]. NF-κB p65 in the cytoplasm binds to its inhibitor IκB constitutively. When stimulated by an external source, such as LPS, I-κB is phosphorylated and degraded, and the NF-κB/IκBα complex dissociates, thus activating NF-κB. The activated NF-κB p65 translocates to the nucleus and binds to the promoter or enhancer of the target gene [18, 19]. In the present study, the addition of 25, 50, 75, or 100 μg/mL capsaicin to LPS (1 μg/mL)-induced macrophages significantly reduced the phosphorylation of NF-κB p65 at 24 h, inhibiting the expression of inflammatory mediators, thereby exerting an anti-inflammatory effect.

The results of this study showed that the treatment of the macrophage inflammatory model with 25, 50, 75, or 100 μg/mL capsaicin significantly inhibited the mRNA and protein expression of IL-6, TNF-α, and NO in macrophages at 24 h after LPS (1μg/mL) induction and inhibited the phosphorylation of NF-κB p65 in macrophages. As capsaicin inhibits the inflammatory cytokines IL-6, TNF-α, and iNOS, the inhibition of LPS-induced NF-β pathway activation by capsaicin, which leads to decreased expression of inflammatory cytokines, is one of the important molecular mechanisms underlying its anti-inflammatory effect.

The MAPK pathway is an important inflammatory pathway by which LPS could affect the activation of macrophages. p38 and ERK, two of the most important signal transduction pathway proteins, are involved in the onset and progression of inflammation [20]. Therefore, we hypothesized that the MAPK pathway could contribute to the anti-inflammatory effects of capsaicin.

When macrophages are stimulated by signals, some proteins undergo conformational changes, such as phosphorylation, enter the nucleus, and activate the transcription of target genes, thereby activating macrophages and promoting the secretion of a series of active factors to elicit an immune response [21]. Capsaicin has anti-inflammatory and analgesic effects, but whether the anti-inflammatory effect is related to the inflammatory response or the MAPK signaling pathway remains unclear. Therefore, the effect of capsaicin on the MAPK signaling pathway was evaluated by inducing *in vitro* macrophage inflammation. The results showed that treatment with 25, 50, 75, or 100 μg/mL capsaicin significantly reduced the phosphorylation of ERK42/44 in

macrophages via LPS-induced MAPK activation. This suggested that the anti-inflammatory effect of capsaicin was achieved by regulating the MAPK signaling pathway.

The results of our study showed that the addition of 25, 50, 75, and 100 μg/mL capsaicin significantly inhibited the mRNA and protein expression of IL-6, TNF-α, and NO in macrophages and the phosphorylation of ERK42/44 signaling molecules induced by LPS in mice peritoneal macrophages at 24 h. This suggests that the anti-inflammatory activity of capsaicin may involve the downregulation of phosphorylation of signaling molecules such as ERK42/44 in the MAPK inflammatory signaling pathway in macrophages, thus inhibiting the expression of pro-inflammatory factors such as IL-6, TNF-α, and NO [22].

As a protective defense response, inflammation is a common pathway involved in the occurrence and development of major diseases in humans, such as infections, tumors, cardio-cerebrovascular diseases, senile dementia, neurodegenerative diseases, allergic diseases, and mental disorders. Because the existing synthetic anti-inflammatory drugs are associated with obvious adverse reactions, and there are several Chinese herbal medicines with curative effect and less adverse effects, in this study, using a cell inflammation model, we examined the effect of capsaicin on inflammatory responses in order to provide a basis to develop more effective and safer anti-inflammatory drugs with capsaicin [23]. There has been some progress in research on the anti-inflammatory effects of capsaicin. However, the structural types of its anti-inflammatory active components are diverse and their mechanisms of action are complex, and research efforts in this regard are limited. More reasonable and reliable pharmacological models must be established to carry out in-depth and systematic studies on the mechanism of action of capsaicin [24]. This study is the first to systematically examine the anti-inflammatory activity of capsaicin. However, these experiments were all carried out *in vitro*. In the future, the anti-inflammatory effects of capsaicin and its mechanism of action should be examined *in vivo*.

In our *in vitro* model of macrophage inflammation, capsaicin significantly reduced the gene and protein expression of IL-6, TNF-α, and NO. A potential mechanism underlying this anti-inflammatory action is reduced phosphorylation of NF-κB p65, inhibition of NF-κB p65 translocation into the nucleus, and phosphorylation of ERK42/44 in the upstream NF-κB signaling pathway.

In this study, we developed an LPS-induced mouse abdominal cavity macrophage inflammatory model and used cell biology and biochemistry technologies to study the effect of capsaicin on the secretion of inflammatory factors and the mechanism of action. Screening, toxicity evaluation, and cellular and molecular pharmacology are important to promote the application of capsaicin [25].

In the future, we aim to perform clinical and *in vivo* pharmacological studies to determine whether capsaicin is safe and effective. Capsaicin has been used in the treatment of chronic neuralgia, which is difficult to treat. However, due to the associated irritation, the applications of capsaicin are limited [26]. Because capsaicin has strong analgesic and anti-inflammatory effects, it is of significance to understand how to modify its molecular structure and to develop new capsaicin preparations for promoting the application of capsaicin in the pharmaceutical industry [27].

Electronic supplementary material

The electronic supplementary material (ESM) is available with the online version of the article at https://doi.org/10.1024/0300-9831/a000721

ESM 1. Western blot of actin-(1) (Figure E1), Western blot of erk3 (Figure E2), Western blot of p-Erk (Figure E3), Western blot of p65-2 (Figure E4), Western blot of p-p65 (Figure E5), Primer sequences (Table E1), Effects of capsaicin on the phagocytic function of peritoneal macrophages (Table E2)

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Conflict of interest
The authors declare that there are no conflicts of interest.

Authorship
JL and HW contributed equally to this work. JL and HW conducted most of the experiments and drafted the manuscript. LZ performed the remaining experiments. NA, WN, and QG analyzed the data. YY conceived and designed the study, supervised the experiments, and was responsible for the decision to submit the article for publication. All authors read and approved the final manuscript.

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