

The NK-1R Antagonist Aprepitant Prevents LPS-Induced Oxidative Stress and Inflammation in RAW264.7 Macrophages

This article was published in the following Dove Press journal:
Drug Design, Development and Therapy

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Background: The macrophage is one of the most important types of immune cells that protect against harmful stimuli. Macrophage activation plays a pivotal role in the progression and development of various inflammatory diseases. The neurokinin 1 receptor (NK-1R) is a G protein-coupled receptor that plays an important role in inflammatory diseases. Aprepitant is a kind of NK-1R antagonist. The purpose of this study is to determine the protective effect of aprepitant in lipopolysaccharide (LPS)-induced inflammatory responses in macrophages.

Methods: We examined the anti-inflammatory and anti-oxidant effects of aprepitant in LPS-treated RAW264.7 macrophages by using real-time PCR, ELISA, and Western blot analysis. We also assessed cellular oxidative stress signaling by measuring the levels of cellular MDA, total ROS, and NADPH oxidase expression. Cellular NO production was measured by DAF-FM DA staining. The inhibitory effect of aprepitant against NF- κ B signaling was evaluated by luciferase assay and Western blot analysis.

Results: The expression of NK-1R is increased in LPS-induced macrophages, suggesting a potential role of the receptor in the inflammatory response. We show that aprepitant protects macrophages against oxidative stress by reducing the generation of ROS and the expression of NOX-4. Furthermore, aprepitant inhibits the secretion of pro-inflammatory cytokines and chemotactic factors by mediating the NF- κ B signaling pathway.

Conclusion: The NK-1R receptor antagonist aprepitant acts as an anti-inflammatory agent, indicating that the blockage of the NK-1R pathway in macrophages has the potential to suppress inflammation.

Keywords: aprepitant, NK-1R, inflammation, NF- κ B, oxidative stress

Introduction

Inflammatory response is a pathophysiological process against harmful stimuli called by pathogen infection or tissue damage.¹ And it is well known that inflammation plays a vital role in various diseases such as cardiovascular disease, diabetes, and even cancers.² Macrophages, which comprise a major component of the immune cell population, take an important part in innate immunity. Recent studies have corroborated macrophages participate in anti-inflammatory processes.³ Lipopolysaccharide (LPS) is a main component of the membrane from Gram-negative bacteria. It is well known that LPS can induce macrophages to differentiate into two kinds of phenotype – M1 and M2, which play different roles in the inflammation responses.⁴ Activated macrophages release various pro-inflammatory cytokines and chemotactic factors, such as tumor necrosis factor- α (TNF- α), interleukins (ILs),⁵ and matrix

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metalloproteinases (MMPs). And this process is upregulated by activating nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs) signaling pathways.⁶ Blockage of aberrant macrophage activation may become a promising therapeutic strategy in treating inflammatory disorders.

Neurokinin 1 receptor (NK-1R), a seven-transmembrane domain G-protein-coupled receptor, is an important member of three receptor hypotypes for tachykinins, regulates the function of the neuropeptide substance P (SP).⁷ NK-1R spreads widely in the nervous system and immune cells of respiratory and digestive tracts.⁸ The previous studies have corroborated that NK1R participates in important pathological and physiological processes including pain, inflammation, smooth muscle contraction, osteoblast differentiation, and intestinal fibrosis of colitis.^{9,10} Furthermore, the upregulation of NK1R has been found in malignant tumors such as malignant glioma,¹¹ pancreatic cancer,¹² and thyroid cancer. Aprepitant is a kind of neurokinin 1 receptor (NK-1R) antagonist permitted to impede vomiting and nausea caused by chemical therapy.¹³ As an NK-1R antagonist, aprepitant is safe and well tolerated for clinical use in most of the population.¹⁴ Furthermore, aprepitant has displayed a powerful anti-inflammatory capacity by suppressing the expression of chemokines and cytokines.¹⁵ However, the pharmacological function of aprepitant in LPS-induced macrophage activation has been less reported. The purposes of our study are to determine the anti-inflammatory effects of aprepitant in LPS-induced inflammatory responses in RAW264.7 macrophages.

Materials and Methods

Cell Culture and Treatment

RAW264.7 macrophages, acquired from the American Type Culture Collection (Manassas, USA), were maintained in

Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a 5% CO₂ atmosphere. Cells were treated with 1 μ g/mL LPS in the presence or absence of aprepitant (5, 10 μ M) for 24 h.

Real-Time PCR Analysis

Total RNA was separated from R264.7 macrophages using the Qiazol (Qiagen, USA). cDNA was synthesized from total RNA using RT-PCR using a commercial cDNA synthesis kit (Bio-Rad, USA). One microgram of cDNA was used to measure the expression of target genes using a SYBR Green Master mix (Bio-Rad, USA). The relative levels of target gene were quantified by normalized to GAPDH using a comparative $2^{-\Delta\Delta CT}$ method and expressed as the fold induction. Primer sequences used for real-time PCR are shown in Table 1.

Western Blot Analysis

Cells cultured on 6-well plate were lysed with protein lysis buffer and broken by ultrasound. The supernatant was taken and the total protein concentration was measured by a BCA method. Separated by 100 g/L polyacrylamide gel electrophoresis, the supernatant was transferred to the nitrocellulose membrane with the 50 g/L skim milk powder chamber for 1 h. The membrane was washed and sequentially loaded with primary overnight at 4°C and secondary antibodies for 2 h at room temperature. The reaction was followed by 5 min after the addition of the chemiluminescence reagent, then exposed to X film, and the KODAK image was used to take analysis.¹⁶

Intracellular ROS Detection

Total cellular reactive oxygen (ROS) was measured to reflect the overall status of oxidative stress. In brief, the intracellular ROS level was determined by staining

Table 1 The Primers Sequences

Target Gene	Upstream Sequence (5'-3')	Downstream Sequence (5'-3')
NK-1R	5'-GCTCTGTGCATGGGTCTCTT-3'	5'-AGGAAGGATGGCTCCAGGAT-3'
NOX-4	5'-TGAACACAGTGAAGATTTCTTGAAC-3'	5'-GACACCCGTCAGACCAGGAA-3'
NOX-2	5'-ACTCCTTGGAGCACTGG-3'	5'-GTTCTGTCCAGTTGCTTTCG-3'
COX-2	TCTC 5'-CAGACAACATAAAGTCGCGCTT-3'	5'-GATACACCTCTCCACCAATGACC-3'
iNOS	5' C 5'-GAAACGCTTCACTTCCAA-3'	5'-TGAGCCTATATTGCTGTGGCT-3'
TNF- α	5'-5T 5'-ACTGAAGTTCGGGGTGATTGGTCC-3'	5'-CAGCCTTGTCCTTGAAGAGAACC-3'
MCP-1	5' 5'-GCATCCACGTGTTGGCTCA-3'	5'-CTCCAGCCTACTCATTGGGATCA-3'
MMP-2	5'-CGATGTCGCCCCATAAACAG-3'	5'-GCATGGTCTCGATGGTGTTC-3'
MMP-9	GAA5 5'-AAGGGTACAGCC TGTTCTCTGGT-3'	5'-CTGGATGCCGCTCTAT GTCGCTCT-3'
GAPDH	5'-CCGTGAAAAGAT GACCCAG-3'	5'-TAGCCACGCTCGGTC AGG-3'

RAW264.7 cells with the ROS probe dihydroethidium (DHE). After washing 3 times, the cells were incubated with serum-free medium containing 5 μ M DHE (Thermo Fisher Scientific, USA) for 30 min at a temperature of 37°C in darkness.¹⁶ The cells were then washed 3 times with PBS buffer, and the resulting fluorescent images were visualized using a fluorescence microscope. Image J software was used to analyze the fluorescence intensity to determine the concentration of ROS.

Determination of Malondialdehyde (MDA)

Levels of intracellular MDA in RAW264.7 cells were assessed to index lipid peroxidation based on a modification of the method described before.¹⁷ Cells were homogenized, followed by centrifugation at 1500 \times g for 10 min. Then, the cell lysis was added to a reaction mixture. After boiling for 1 h at 95°C and centrifugation at 3000 \times g for 10 min, OD value at 550 nm was recorded to index intracellular MDA.

Measurement of Nitric Oxide (NO)

Intracellular levels of NO were measured using DAF-FM staining approach as reported before.¹⁸ After treatment, cells were washed and loaded with 5 μ M DAF-FM and incubated at 37°C for 20 min. After incubation, cells were washed three times by PBS at pH7.4. A confocal microscope equipped was used to assess the stained cells.

ELISA

Cells were treated with 1 μ g/mL LPS in the presence or absence of aprepitant (5, 10 μ M) for 24 h. We adopted the procedure of ELISA as reported before with a slight modification.¹⁸ In brief, cultured media were harvested to measure secreted levels of TNF- α , MCP-1, and PGE2. Cell lysates were used to measure MMP-2 and MMP-9

using commercial ELISA kits (R&D Systems, Beijing, China) according to the manufacturers' instructions.

Luciferase Activity of NF- κ B

RAW264.7 macrophage cells were cultured in a 12-well plate for 18 h. Cells were then transfected with plasmids containing NF- κ B using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h, cells were treated with 1 μ g/mL LPS in the presence or absence of aprepitant (5, 10 μ M) for 6 h. The luciferase activity of NF- κ B was carried out using the commercial kit from Promega, USA according to the manufacturer's instruction.¹⁹

Statistical Analysis

All results were presented as the mean \pm SD. The differences were performed by the one-way ANOVA. Significance was defined as P-values < 0.05.

Results

LPS Increases the Expression of NK-1R in Macrophages

To explore the function of aprepitant, we first treated macrophages with LPS and then measured the expression of NK-1R. As shown in Figure 1A, the mRNA levels increased to roughly 2.3-, 3.1-, and 4.2-fold in response to stimulation with LPS (0.5, 1, 2 μ g/mL) for 24 h. Meanwhile, the three doses of LPS increased the protein levels of NK-1R to 1.9-, 2.7-, and 3.6-fold (Figure 1B). Therefore, we confirmed that LPS increased both protein and mRNA levels of NK-1R in macrophages.

Aprepitant Protects Macrophages Against LPS-Induced Oxidative Stress

Intracellular ROS and MDA are typical indicators of oxidative stress. To examine the effects of aprepitant

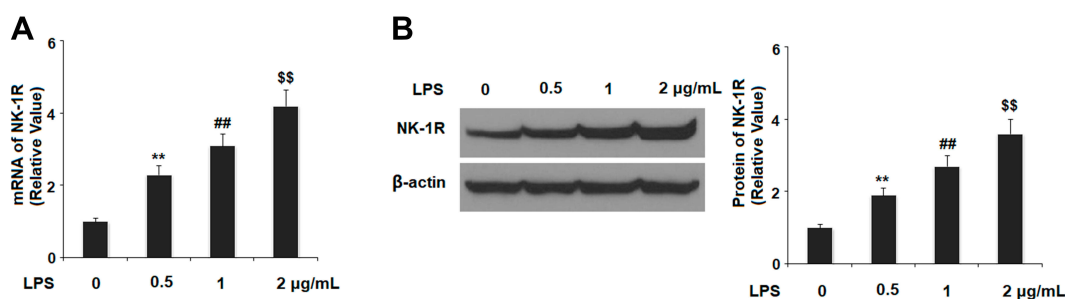


Figure 1 LPS increased the expression of NK-1R in RAW264.7 macrophages. Cells were treated with LPS (0.5, 1, 2 μ g/mL) for 24 h. (A) mRNA of NK-1R; (B) protein of NK-1R (**, ##, \$\$, P<0.01 vs the control group, the 0.5 μ g/mL LPS group, the 1 μ g/mL LPS group, respectively).

on oxidative stress, we measured on intracellular ROS and MDA levels. As shown in Figure 2A, intracellular ROS was increased by treatment with 1 $\mu\text{g/mL}$ LPS to approximately 4.5-fold, which was significantly rescued by the two doses of aprepitant to only 2.6- and 1.5-fold, respectively. The MDA level increased to 2.3-fold by exposure to LPS. However, two doses of aprepitant decreased the MDA levels to 1.7- and 1.3-fold, respectively (Figure 2B). Macrophages treated with LPS alone resulted in high expression of NOX-2 and NOX-4 at both the mRNA (Figure 3A) and protein levels (Figure 3B), which were suppressed by aprepitant in a dose-dependent manner. Taken together, our

data indicated that aprepitant suppressed LPS-induced oxidative stress.

Aprepitant Inhibits Expression of COX-2 and Secretion of PGE₂ Induced by LPS

To investigate the effects of aprepitant on the expression of COX-2 and secretion of PGE₂ induced by LPS, macrophages were pretreated with 5 and 10 μM aprepitant for 24 h, followed by stimulation with LPS. As shown in Figure 4A and B, the mRNA expression and protein secretion of COX-2 reduced to 4.3- and 3.4-fold. However, the two doses of aprepitant decreased the mRNA expression of COX-2 to 2.7- and 1.5- fold,

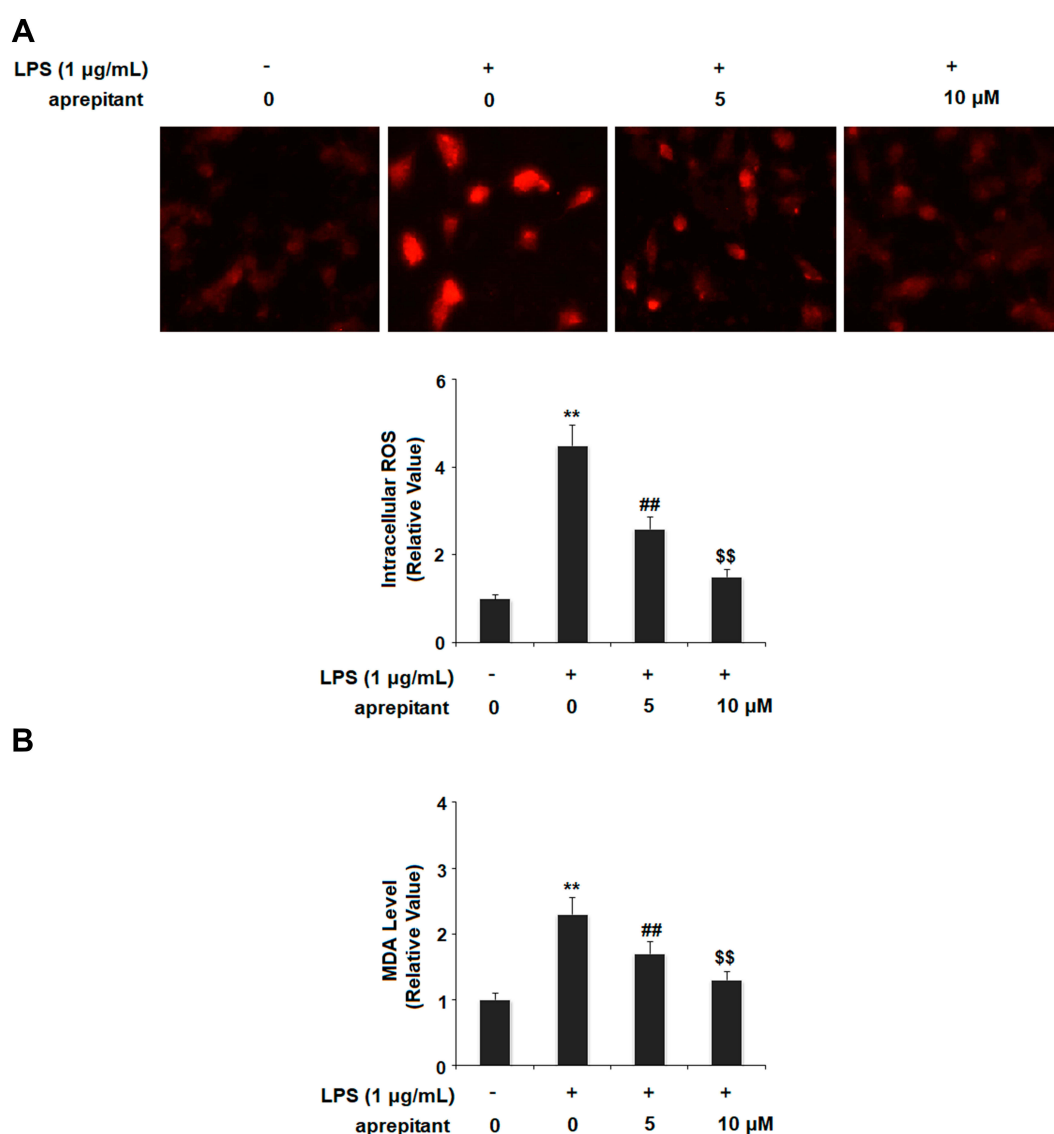


Figure 2 Aprepitant prevented LPS-induced oxidative stress in RAW264.7 macrophages. Cells were treated with 1 $\mu\text{g/mL}$ LPS in the presence or absence of aprepitant (5, 10 μM) for 24 h. (A) Intracellular ROS was determined by dihydroethidium (DHE) staining. (B) The levels of malondialdehyde (MDA) were measured (**, ##, \$\$, $P < 0.01$ vs the control group, the 1 $\mu\text{g/mL}$ LPS group, the 1 $\mu\text{g/mL}$ LPS+5 μM aprepitant group, respectively).

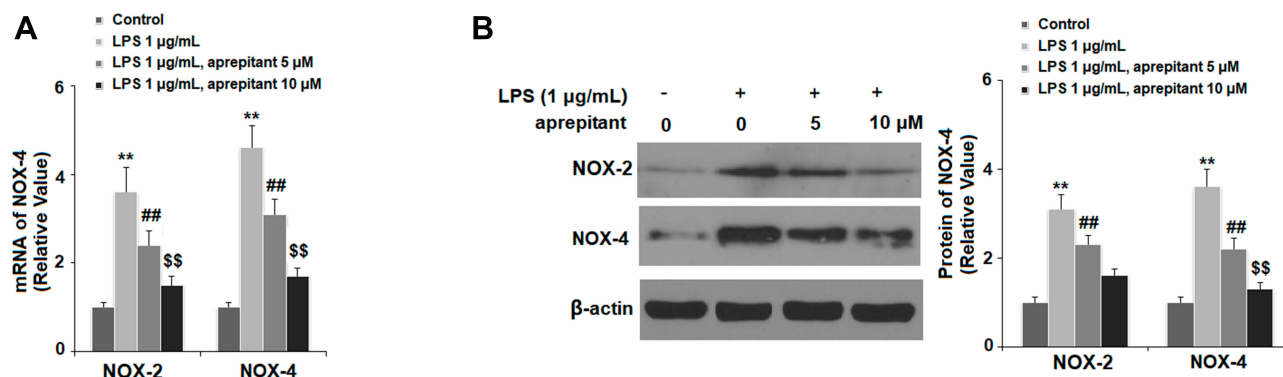


Figure 3 Aprepitant prevented LPS-induced expression of NOX-2 and NOX-4 in RAW264.7 macrophages. Cells were treated with 1 μ g/mL LPS in the presence or absence of aprepitant (5, 10 μ M) for 24 h. (A) mRNA of NOX-2 and NOX-4; (B) protein of NOX-2 and NOX-4 as measured by Western blot analysis (**, ##, \$\$, $P < 0.01$ vs the control group, the 1 μ g/mL LPS group, the 1 μ g/mL LPS+5 μ M aprepitant group, respectively).

while the secretion of protein was reduced to 2.2- and 1.4-fold, respectively. Concordantly, exposure to LPS alone increased the production of PGE_2 to 1533.7 pg/mL, which was ameliorated by treatment with the two doses of aprepitant to concentrations of only 885.3 and 566.4 pg/mL (Figure 4C).

Aprepitant Prevents Expression of iNOS Induced by LPS in Macrophages

To determine the effects of aprepitant on the production of iNOS and NO induced by LPS, real-time PCR and Western blot analysis were employed. As shown in Figure 5A, the mRNA expression of iNOS was increased to 3.8-fold by

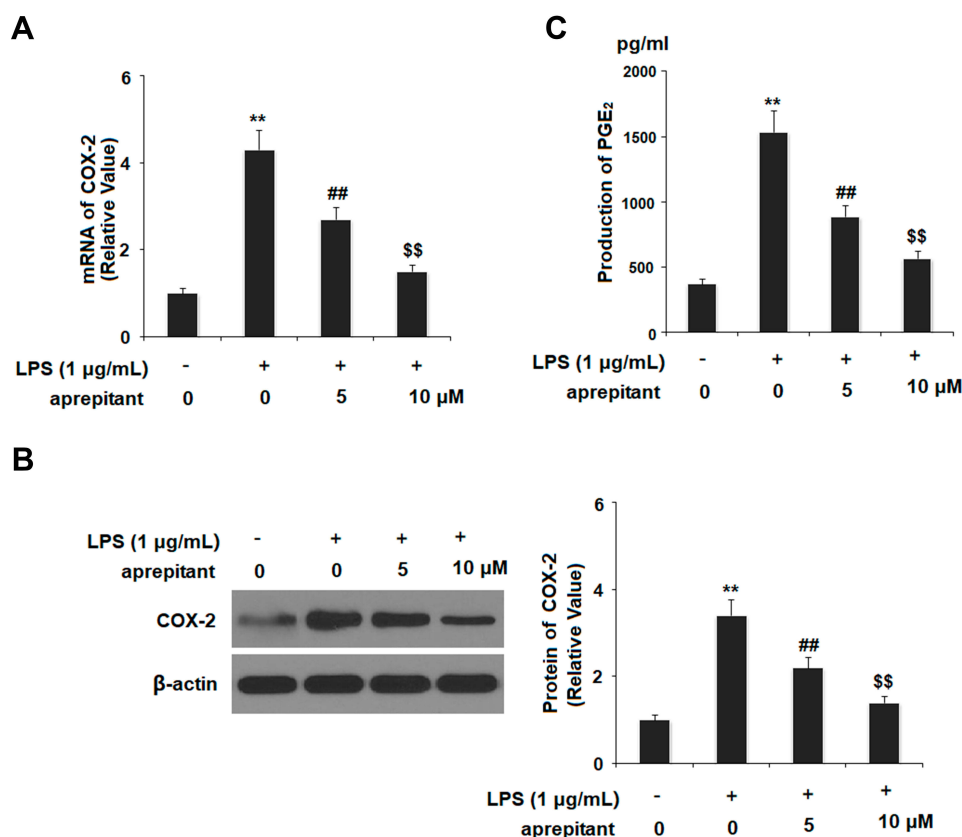


Figure 4 Aprepitant prevented LPS-induced expression of cyclooxygenase 2 (COX-2) and secretion of prostaglandin E2 (PGE_2) in RAW264.7 macrophages. Cells were treated with 1 μ g/mL LPS in the presence or absence of aprepitant (5, 10 μ M) for 24 h. (A) mRNA of COX-2; (B) protein of COX-2; (C) production of PGE_2 as measured by ELISA (**, ##, \$\$, $P < 0.01$ vs the control group, the 1 μ g/mL LPS group, the 1 μ g/mL LPS+5 μ M aprepitant group, respectively).

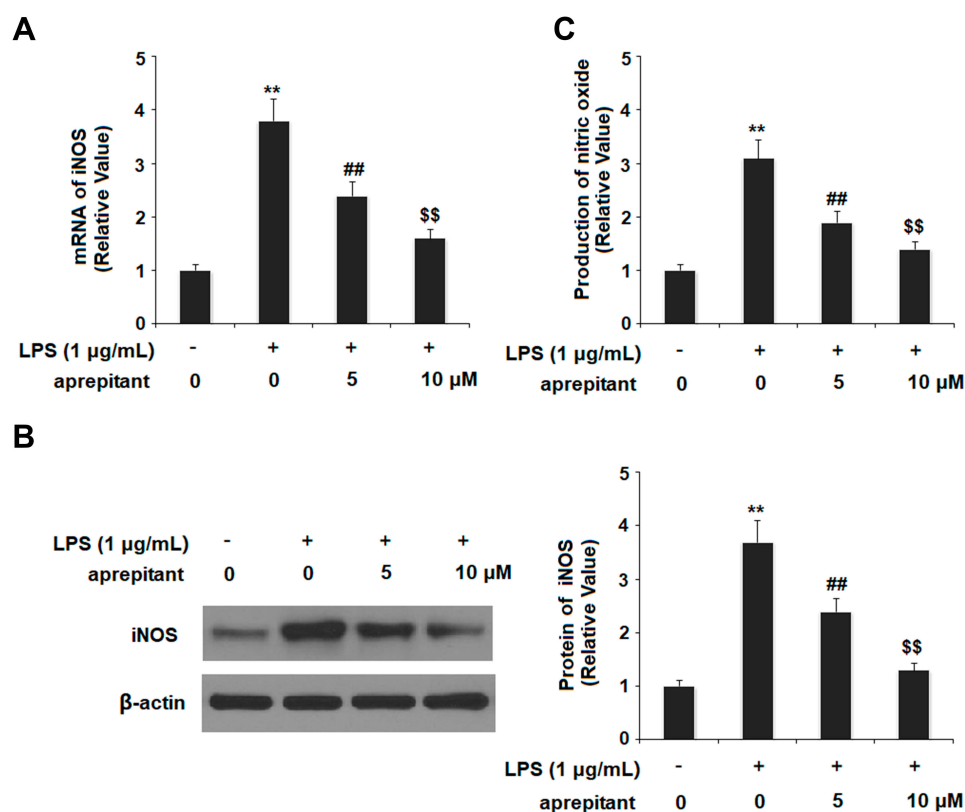


Figure 5 Aprepitant prevented LPS-induced expression of inducible nitric oxide synthase (iNOS) in RAW264.7 macrophages. Cells were treated with 1 µg/mL LPS in the presence or absence of aprepitant (5, 10 µM) for 24 h. (A) mRNA of iNOS; (B) protein level of iNOS as measured by Western blot analysis; (C) production of nitric oxide (NO) (**, ##, \$\$, $P < 0.01$ vs the control group, the 1 µg/mL LPS group, the 1 µg/mL LPS+5 µM aprepitant group, respectively).

exposure to LPS, which was decreased by treatment with 5 and 10 µM aprepitant to only 2.4- and 1.6-fold. At the protein level, LPS increased iNOS expression to 3.1-fold, which was decreased to 1.9- and 1.4-fold by aprepitant (Figure 5B). As shown in Figure 5C, NO production increased to 3.7-fold upon exposure to LPS, which was reduced to only 2.4 and 1.3-fold by the two doses of aprepitant.

Aprepitant Suppresses LPS-Induced Expression of Pro-Inflammatory Cytokines

When exposed to LPS alone, the mRNA expression of TNF- α was upregulated approximately 5.3-fold, while the protein secretion of TNF- α increased from 267.8 to 2098.9 pg/mL. Meanwhile, the mRNA of MCP-1 increased to 7.5-fold, protein secretion of MCP-1 was increased from 336.9 to 3058.7 pg/mL. However, these upregulations were significantly ameliorated by treatment with the two doses of aprepitant. The results in Figure 6A showed that mRNA levels of TNF- α were decreased to 3.2- and 1.9- fold, while MCP-1 expression was reduced to 4.5- and 2.3-fold, respectively. The results of Figure 6B showed that treatment with the two

doses of aprepitant ameliorated protein secretion of TNF- α and MCP-1, reducing the protein concentration of TNF- α to 1566.5 and 1045.5, and that of MCP-1 to only 1877.2 and 1366.7 pg/mL, respectively. Thus, aprepitant is considered to have a potent anti-inflammatory effect.

Aprepitant Reduces LPS-Induced Expression of MMPs

To determine whether aprepitant treatment affects the expression of MMPs in macrophages induced by LPS, we investigated the expression of MMP2 and MMP9. The results in Figure 7A showed that the mRNA levels of MMP-2 and MMP-9 upregulated to 4.1- and 4.7-fold. Respectively, upon exposure to LPS alone. However, these levels were reduced to 2.9-, and 3.2-fold by 5 µM of aprepitant, respectively, while the dose of 10 µM further regulated these enzymes to only 2.3-, and 2.1-fold. The results in Figure 7B showed that the protein levels of MMP-2 and MMP-9 increased from 103.5 and 153.6 pg/mL to 1366.4 and 1988.3 pg/mL, respectively, upon exposure to LPS. The 5 µM aprepitant decreased these numbers to 923.6 and

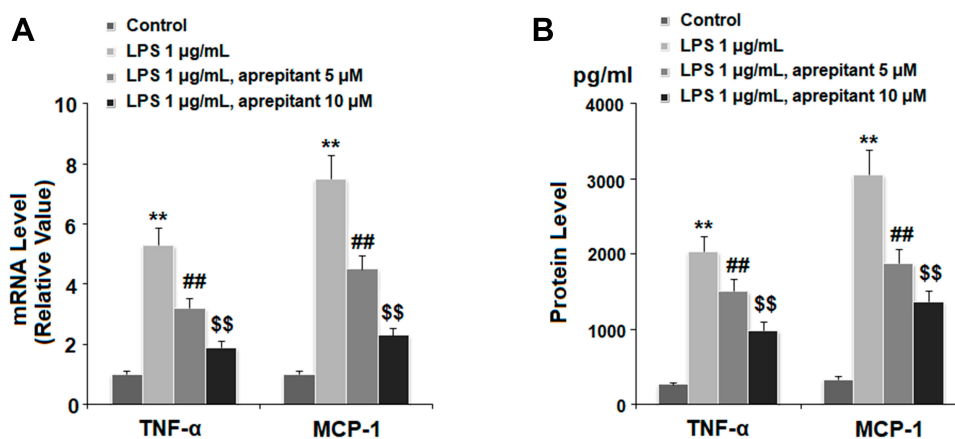


Figure 6 Aprepitant reduced LPS-induced expression of pro-inflammatory cytokines in RAW264.7 macrophages. Cells were treated with 1 μg/mL LPS in the presence or absence of aprepitant (5, 10 μM) for 24 h. (A) mRNA of TNF-α and MCP-1; (B) protein of TNF-α and MCP-1 as measured by ELISA (**, ##, \$\$, $P < 0.01$ vs the control group, the 1 μg/mL LPS group, the 1 μg/mL LPS+5 μM aprepitant group, respectively).

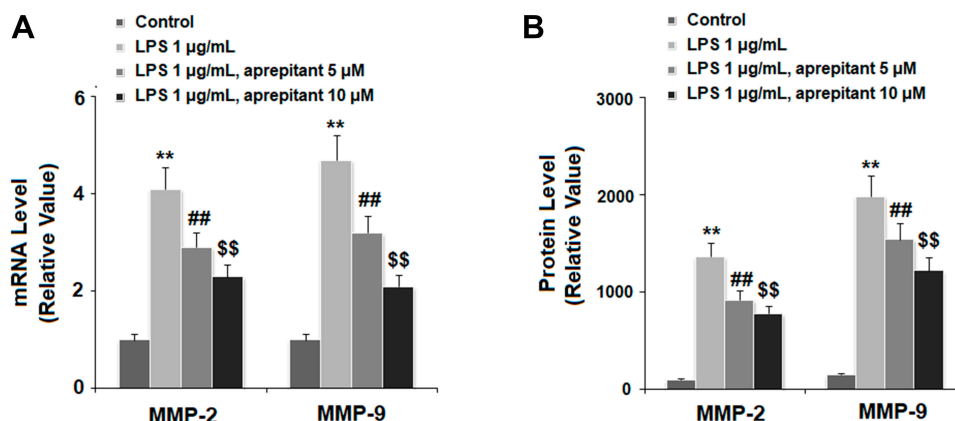


Figure 7 Aprepitant reduced LPS-induced expression of matrix metalloproteinases (MMPs) in RAW264.7 macrophages. Cells were treated with 1 μg/mL LPS in the presence or absence of aprepitant (5, 10 μM) for 24 h. (A) mRNA of MMP-2 and MMP-9; (B) protein of MMP-2 and MMP-9 (**, ##, \$\$, $P < 0.01$ vs the control group, the 1 μg/mL LPS group, the 1 μg/mL LPS+5 μM aprepitant group, respectively).

1544.5 pg/mL, while the dose of 10 μM further reduced the productions to only 782.9 and 1222.6 pg/mL, respectively.

Aprepitant Mitigates Activation of NF-κB

To determine the effects of aprepitant treatment on LPS-induced activation of NF-κB, we measured nuclear translocation of p65 and luciferase activity of NF-κB. As shown in Figure 8A, nuclear translocation of NF-κB p65 was increased to approximately 4.2-fold by treatment with LPS, which was significantly rescued by the two doses of aprepitant to only 2.7- and 1.6-fold, respectively. Then, we investigated the luciferase activity of NF-κB. Treatment with LPS increased it to 533.8-fold, which was rescued by the two doses of aprepitant to 366.8- and 125.6-fold, respectively (Figure 8B). The results indicated that aprepitant treatment can mitigate the activation of NF-κB.

Discussion

Chronic inflammation is testified to associate with the generation and development of cancer.²⁰ For example, ulcerative colitis, which is a kind of chronic inflammation caused by intestinal flora, is clearly bound with the generation and development of colon cancer.²¹ Moreover, overexpression of NK-1R has been reported in the rectum and colon of patients with inflammatory bowel diseases,²² as well as in malignant glioma,¹¹ pancreatic cancer,¹² and thyroid cancer. These established cases built a relationship between NK-1R and inflammation. In our experiment, we used aprepitant to determine whether NK-1R antagonists affect the inflammatory response. As macrophages have been corroborated to play a key role in anti-inflammatory processes,³ we chose RAW264.7 macrophages for this experimentation.

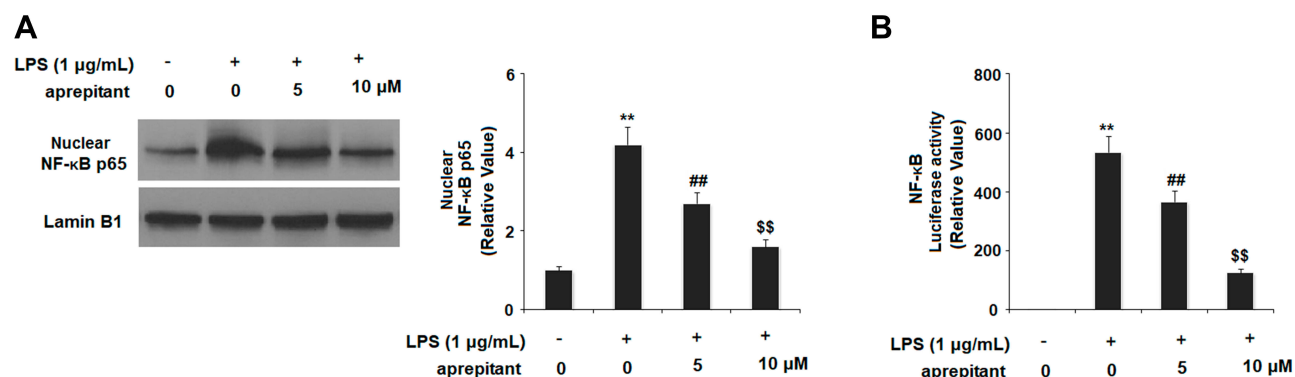


Figure 8 Aprepitant prevented activation of NF-κB in RAW264.7 macrophages. Cells were treated with 1 μg/mL LPS in the presence or absence of aprepitant (5, 10 μM) for 6 h. (A) Nuclear translocation of NF-κB p65 with Lamin B1 as an internal control; (B) luciferase activity of NF-κB (*, **, ##, \$\$, $P < 0.01$ vs the control group, the 1 μg/mL LPS group, the 1 μg/mL LPS+5 μM aprepitant group, respectively).

Oxidative stress refers to the imbalance between oxidation and anti-oxidation in vivo. It has been confirmed that oxidative stress can lead to cell apoptosis,²³ and is common in inflammation. Aprepitant treatment decreased the levels of ROS and MDA obviously. These findings suggested that aprepitant could protect macrophages by inhibiting oxidative stress. Furthermore, activated macrophages induced by LPS released various pro-inflammatory cytokines and chemokines²⁴ such as MCP-1 and TNF-α. Excessive production of these factors takes an important part in affecting the development of inflammation. TNF-α is described as a primary inflammatory regulator in the pathogenesis of inflammation. For example, TNF-α is consumedly increased in synovial tissue in rheumatoid arthritis (RA).²⁵ Interestingly, overexpression of NOX-4 has also been associated with the upregulation of TNF-α.²⁶ In this study, the expression of these pro-inflammatory factors was decreased significantly by aprepitant. In addition, LPS stimulation activates COX-2 and iNOS transcription which lead to the overexpression of PGE₂ and NO in macrophages, respectively.²⁷ These inflammatory mediators are highly increased in inflammation.²⁸ The results in this study show that aprepitant reduced PGE₂ and NO production induced by LPS, due to its inhibition on the production of COX-2 and iNOS.

NF-κB-dependent signaling pathway is a key modulator in the development of inflammation. The LPS stimulation triggered the activation of NF-κB pathway. The phosphorylation of IκBα by upstream kinases is a critical process for the activation of NF-κB.²⁹ NF-κB activation upregulated the expression of pro-inflammatory cytokines including TNF-α and MCP-1.³⁰ Furthermore, NF-κB

modulated the production of PGE₂ and NO via regulating the expression of regulatory enzymes COX-2 and iNOS. NF-κB pathway has been considered as an important target for the treatment of inflammation. Our data showed that aprepitant inhibited the activation of NF-κB via modulating phosphorylation of IκBα.

Aprepitant treatment reversed substance P- and CCL5-mediated monocyte chemotaxis by interacting with a specific NK-1R isoform,³¹ suggesting that substance-dependent NK-1R signaling regulates monocyte function. Our study shows that LPS treatment directly induces the expression of NK-1R, indicating that LPS stimuli could activate NK-1R signaling in macrophages. Furthermore, we show that aprepitant-mediated blockage of NK-1R suppresses LPS-induced inflammation. These data indicate that aprepitant-mediated NK-1R inhibition in myeloid cells could occur through a substance P-dependent or -independent mechanism. Recently, NK-1R has been shown to influence the early fever response induced by LPS in mice.³² The blockage of the NK-1R pathway significantly attenuated LPS-induced systematic inflammation in vivo.³³ Thus, NK-1R signaling is an important regulatory pathway of LPS stimuli.

In conclusion, this study investigates the effect of aprepitant in LPS-induced macrophages. The results show that aprepitant inhibits the oxidative stress via suppressing ROS and MDA. Furthermore, aprepitant decreases the production of pro-inflammatory cytokines and chemokines, reduces the expression of regulatory enzymes. In addition, aprepitant inhibits the activation of NF-κB pathway, which regulates the production of inflammatory factors mentioned above. Our data indicate that NK-1R antagonist such as aprepitant is a potent anti-inflammatory agent in vitro cultured macrophages.

Ethical Statement

RAW264.7 macrophages were acquired from the American Type Culture Collection (Manassas, USA). Experimental protocols were approved by Jilin University.

Disclosure

The authors report no conflicts of interest in this work.

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