



2-Methyl Nonyl Ketone From *Houttuynia Cordata Thunb* Alleviates LPS-Induced Inflammatory Response and Oxidative Stress in Bovine Mammary Epithelial Cells

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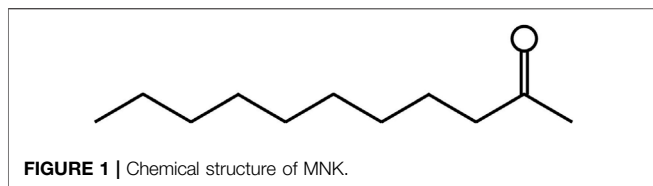
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Mastitis is one of the most common diseases in dairy cows, causing huge economic losses to the dairy industry every year. *Houttuynia Cordata Thunb* (**H.cordata**) is a traditional Chinese herbal medicine that is widely used in clinical treatment. However, the therapeutic effect of 2-methyl nonyl ketone (**MNK**), the main volatile oil component in the aqueous vapor extract of *H. cordata*, on mastitis has been less studied. The purpose of this study was to investigate the protective effect and mechanism of MNK against lipopolysaccharide (**LPS**)-induced mastitis *in vitro*. The results showed that MNK pretreatment of the bovine mammary epithelial cell line (**MAC-T**) enhanced cell viability and inhibited LPS-induced reactive oxygen species (**ROS**) production and inflammatory response. MNK reduced the production of pro-inflammatory cytokines such as interleukin (**IL**) and tumor necrosis factor- α (**TNF- α**) by repressing LPS-induced activation of Toll-like receptor 4-nuclear factor- κ B (**TLR4-NF- κ B**) signaling pathway. In addition, MNK protected cells from inflammatory responses by blocking the downstream signaling of inflammatory factors. MNK also induced *Heme Oxygenase-1* (**HO-1**) production by Nuclear factor erythroid 2-related factor 2 (**Nrf2**) pathway through AKT and extracellular signal-regulated kinase (**ERK**) pathways, thereby reducing LPS-induced oxidative damage for MAC-T cells. In conclusion, MNK played a protective role against LPS-induced cell injury. This provides a theoretical basis for the research and development of MNK as a novel therapeutic agent for mastitis.

Keywords: 2-methyl nonyl ketone, mastitis, inflammation, oxidative stress, LPS

INTRODUCTION

Mastitis is a common infectious disease in dairy cows and is highly influential and easily recurring (Klaas and Zadoks, 2018). Mastitis leads to reduced milk production, lower milk quality, shorter productive life and higher culling rates in dairy cows, causing huge economic losses to dairy farming worldwide (Aghamohammadi et al., 2018; Hadrich et al., 2018; Puerto et al., 2021). Mastitis is an inflammation of the



breast caused by pathogenic microorganisms invading the mammary gland and release large amounts of toxins (Klaas and Zadoks, 2018). LPS is a major component of Gram-negative bacteria and can cause severe mammary immune responses. Therefore, LPS is commonly used to construct models of inflammatory response (Guo et al., 2019). The traditional therapy of treating mastitis is antibiotics, but this is prone to cause drug residues and drug resistance. The development of alternative drugs for the prevention and treatment of mastitis is necessary.

H. cordata has various functions such as anti-inflammatory, antibacterial, antiviral, and immune enhancing (Hemalatha et al., 2014). It is rich in volatile oil, alkaloids, flavonoids and other bioactive substances (Li et al., 2017; Chou et al., 2009). Among the components, the volatile oil of *H. cordata* has the highest medicinal value. The drugs prepared from the volatile oil of *H. cordata* has been used in Asia for the treatment of inflammation-related diseases such as diarrhea, conjunctivitis, and respiratory tract infections (Subhawa et al., 2021). MNK (Figure 1) is soluble in ethanol and oil, but not in water. And it is the most abundant and stable component in the volatile oil of *H. cordata*. MNK has been demonstrated to have anti-inflammatory and antioxidant effects (Wu et al., 2021). However, its anti-inflammatory and antioxidant effects in LPS-induced mammary epithelial cells of cows have not been investigated. In this study, MAC-T cells were selected to simulate the inflammation model of mastitis in cows treated with LPS to investigate the protective effect and mechanism of MNK on LPS-induced MAC-T cell injury in cows. The in-depth study of the efficacy of MNK is beneficial to the quality evaluation and control of *H. cordata* and its preparations, as well as provides a basis for natural or synthetic novel anti-inflammatory drugs.

MATERIALS AND METHODS

Cell Culture and Treatment

The MAC-T was cultured in DMEM/F12 1:1 (Hyclone, South Logan, UT, United States) medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, United States) at 37°C with 5% CO₂. After cells were adhered and full grown to about 90% of the cell culture dish (Corning, NY, United States), the cells were digested with 0.25% trypsin (Gibco, CA, United States), collected cells by centrifugation. And then cells were inoculated into different culture dishes and plates (Corning, NY, United States) at the corresponding density for the different assays.

A stock solution of 1 mg/ml LPS (*Escherichia coli* 0111:B4) was stored at 2–8°C (Sigma-Aldrich, St. Louis, MO, United States). LPS was dissolved in phosphate buffer (Gibco, Grand Island, United States). MNK (Yuanye biomart, Shanghai, China) was

dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO, United States). A new stock solution of 50 mM MNK was stored at 2–8°C. The cells were pre-treated with MNK (at the concentrations of 0, 5, 10, and 20 μM) for 4 h and then incubated with LPS for 24 h. Cells without any treatment were served as a blank control.

Cell Viability Assay

The cell viability of the MAC-T cells was assessed with a cell counting kit-8 (CCK-8 kit, Dojindo, Kumamoto, Japan). The cells were inoculated in 96-well plates (10⁴ cells per well) for 8 h, then treated with MNK or LPS at different levels. After 24 h of treatment, the cells were incubated with 10 μl of CCK-8 solution at 37°C for 1–4 h. The optical density of cells was measured at 450 nm with a microplate reader (TECAN, Safire, Austria). The ratio of optical density values of treated cells to untreated cells was used as cell viability.

Intracellular ROS Measurement

The levels of intracellular ROS were measured by Dihydroethidium (Beyotime Biotech, Haimen, China). MAC-T were seeded in six-well plates at a density of 1×10⁵ cells/well and then treated with LPS or MNK for 24 h. After treatment, the cells were washed with phosphate buffer and then incubated with 0.5 μM DCFH-DA at 37°C for 30 min. After incubation, the cells were washed three times with serum-free DMEMF/12. After resuspension with appropriate DMEMF/12, the fluorescence intensity of the sample was observed under a fluorescence microscope (Olympus Co., Japan). The DHE fluorescence distribution of 10,000 cells was detected using a BD LSR flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

RNA Isolation and Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from cells using a TRIZOL Regent Kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. And RNA concentration was detected by Nanodrop 2000 (Thermo, Massachusetts, United States). Then RNA was reverse transcribed to cDNA using a standard reverse transcription kit (Takara, Tokyo, Japan) according to the instructions. The primers were designed by the NCBI primer design tool, and are shown in Table 1 (synthesized in Comate Bioscience Co. Ltd., Changchun, China). qPCR was performed using the SYBR premix EX Taq (TaKaRa). Relative gene expression was normalized by GAPDH using the 2^{-ΔΔCt} method.

Western Blot Analysis

The MAC-T cells were inoculated at a density of 1×10⁵ cells/well in 6-well plates and then treated with MNK or LPS for 24 h. There were three replicates of each group. Following treatment, cells were harvested and lysed in cold RIPA lysis buffer containing 1 mM PMSF (Beyotime Biotech, Haimen, China). The protein concentrations were assessed with a BCA protein assay kit (Beyotime, Shanghai, china). Equal amounts of protein were separated by 10% SDS-PAGE gels. Western blotting was performed as previously described (Wang et al., 2019).

TABLE 1 | Primers for qPCR.

Primer name	Primer sequence (5-3')	Product length (bp)
<i>GAPDH</i>	Sense Primer: GTTCAACGGCACAGTCAAG Anti-sense Primer: TACTCAGCACCAGCATCAC	117
<i>IL-1β</i>	Sense Primer: CTGTGGAACCAATTTCCGGG Anti-sense Primer: AGGCTTGGTAAAGGACTTGA	60
<i>IL-6</i>	Sense Primer: ATGCTTCCAATCTGGGTTTC Anti-sense Primer: TGAGGATAATCTTTGCGTTC	269
<i>IL-8</i>	Sense Primer: GCTGGCTGTTGCTCTCTTG Anti-sense Primer: GGGTGAAAGGTGTGGAATG	126
<i>TGFβ</i>	Sense Primer: AACCTGTGTTGCTCTCTCGG Anti-sense Primer: GAGGTAGCGCCAGGAATTGT	110
<i>TNF-α</i>	Sense Primer: AGAAGGGAGATCGCCTCAGT Anti-sense Primer: AGACTCGGCATAGTCCAGGT	62
<i>NF-κB P65</i>	Sense Primer: TGGCCCCATATGTGGAGATCA Anti-sense Primer: CTCCTCTCTCCAGGGATGCT	102
<i>NF-κB P50</i>	Sense Primer: TTGACTCAGGTGCAGACGAC Anti-sense Primer: CCCTGTGGCTTTCCAGTTT	101
<i>HO-1</i>	Sense Primer: GGCAGCAAGGTGCAAGA Anti-sense Primer: GAAGGAAGCCAGCCAAGAG	221

Statistical Analysis

All data were analyzed with one-way or two-way ANOVA with Dunnett's multiple comparisons test by using SPSS 22.0 (StatSoft, United States). And the results are expressed as mean values \pm standard deviation (SD). A value of $p < 0.05$ was regarded as statistically significant. $p < 0.01$ was considered extremely significant.

RESULTS

Exogenous MNK Increased Cell Viability in LPS-Induced MAC-T

Firstly, the effects of different concentrations of LPS on the viability of MAC-T cells were determined. As shown from **Figure 2A**, LPS decreased the viability of the MAC-T cells in a concentration-dependent manner. There was an extremely significant decrease at concentrations above 50 μ g/ml. In addition, previous study has reported the inflammatory model *in vitro* with 100 μ g/ml LPS (Sun et al., 2019). Thus 100 μ g/ml was selected as the subsequent treatment concentration. Next, the effects of MNK on the cell viability at 24 h were examined (**Figure 2B**). The results shown that no significant difference was observed at 0.1 or 20 μ g/ml. However, there was an extremely significant increase at concentrations of 1, 10, and 15 μ g/ml. The cell viability was highest at 10 μ g/ml MNK. Then, the effects of MNK on the cell viability with or without LPS at 24 h were examined. It was shown that decreased cell viability induced by LPS was reversed by MNK (**Figure 2C**).

Exogenous MNK Induced ROS Production in LPS-Induced MAC-T

To verify whether the mechanism by which MNK exerts its protective effect is related to ROS, ROS in cells were assayed with Dihydroethidium. In **Figure 3A**, the stronger fluorescence intensity in the LPS group indicated a higher level of intracellular ROS compared to the control group. In contrast, the fluorescence intensity of the LPS plus MNK group was weaker than LPS group,

indicating a lower level of intracellular ROS. Meanwhile, the fluorescence intensity was quantified using a BD LSR flow cytometer. As shown in **Figure 3C**, ROS production of cells treated with 100 μ g/ml LPS was significantly increased compared to the control group. However, ROS production was significantly reduced in the LPS plus MNK group compared to the LPS group.

Effect of MNK on mRNA Levels of Inflammatory Factors Induced by LPS in MAC-T Cells

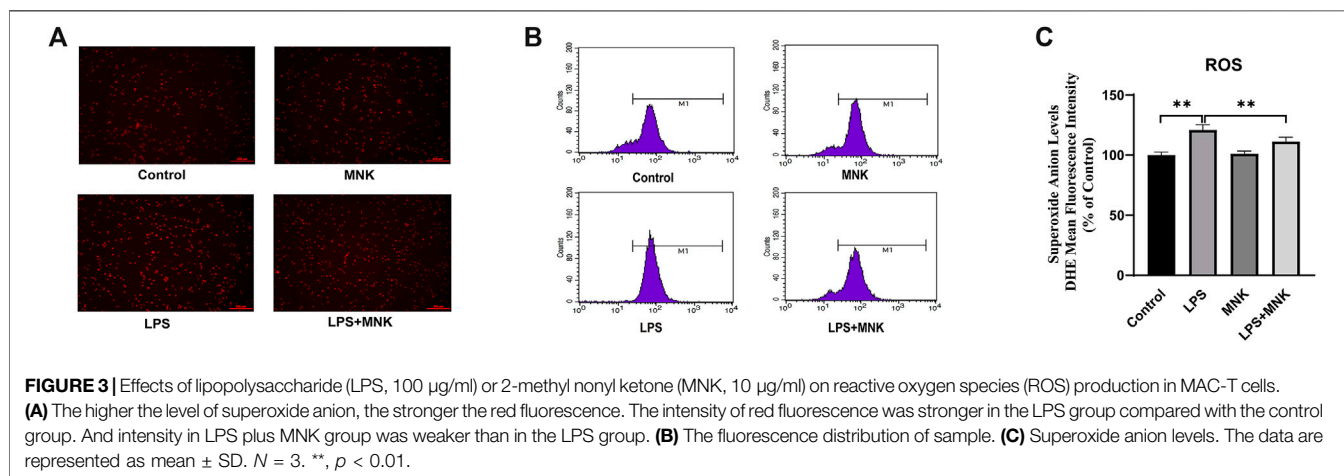
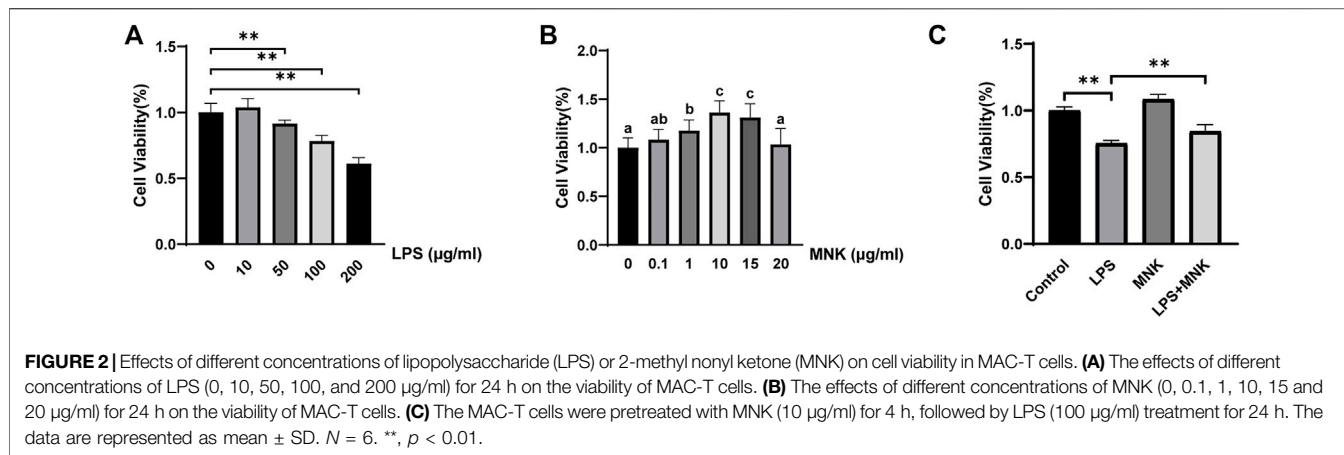
To further analyze the effect of MNK on LPS-induced inflammation in MAC-T cells, the mRNA expression levels of inflammation cytokines were examined by qPCR. As shown in **Figure 4**, it is apparent that LPS significantly increased the mRNA expression of *IL-1 β* , *IL-6*, *IL-8*, *TNF- α* , and *TGF β* . Compared with the LPS group, the mRNA expression of *IL-1 β* , *IL-6*, *IL-8*, *TNF- α* , and *TGF β* was significantly decreased in the LPS plus MNK group.

Effect of MNK on LPS Induced Inflammation-Associated Protein Expression

The effect of MNK on the expression of inflammation-related proteins in LPS-induced MAC-T cells was verified by Western blotting. As shown in **Figure 5**, LPS significantly upregulated the protein expression of signal transducer and activator of transcription (STAT3), IL-6, TNF- α , and TGF β , compared with the control group. In addition, MNK inhibited LPS-induced protein expression of STAT3, IL-6, TNF- α , TGF β and TNF-receptor-associated complex I (TNFR1).

Effect of MNK on TLR4 Signaling Pathway Induced by LPS in MAC-T Cells

TLR4, myeloid differentiation factor 88 (MYD88), p-p65 and p65 protein expression levels were measured by Western blot analysis



to investigate whether the protective effect of exogenous MNK was related to the TLR4 signaling pathway. As shown in **Figure 6**, the mRNA levels of *p65* and *p50* were significantly increased after treatment with LPS, but were reversed by MNK (10 µg/ml) at 24 h in MAC-T cells. In addition, MNK inhibited LPS-induced TLR4, p-p65/p65 protein expression.

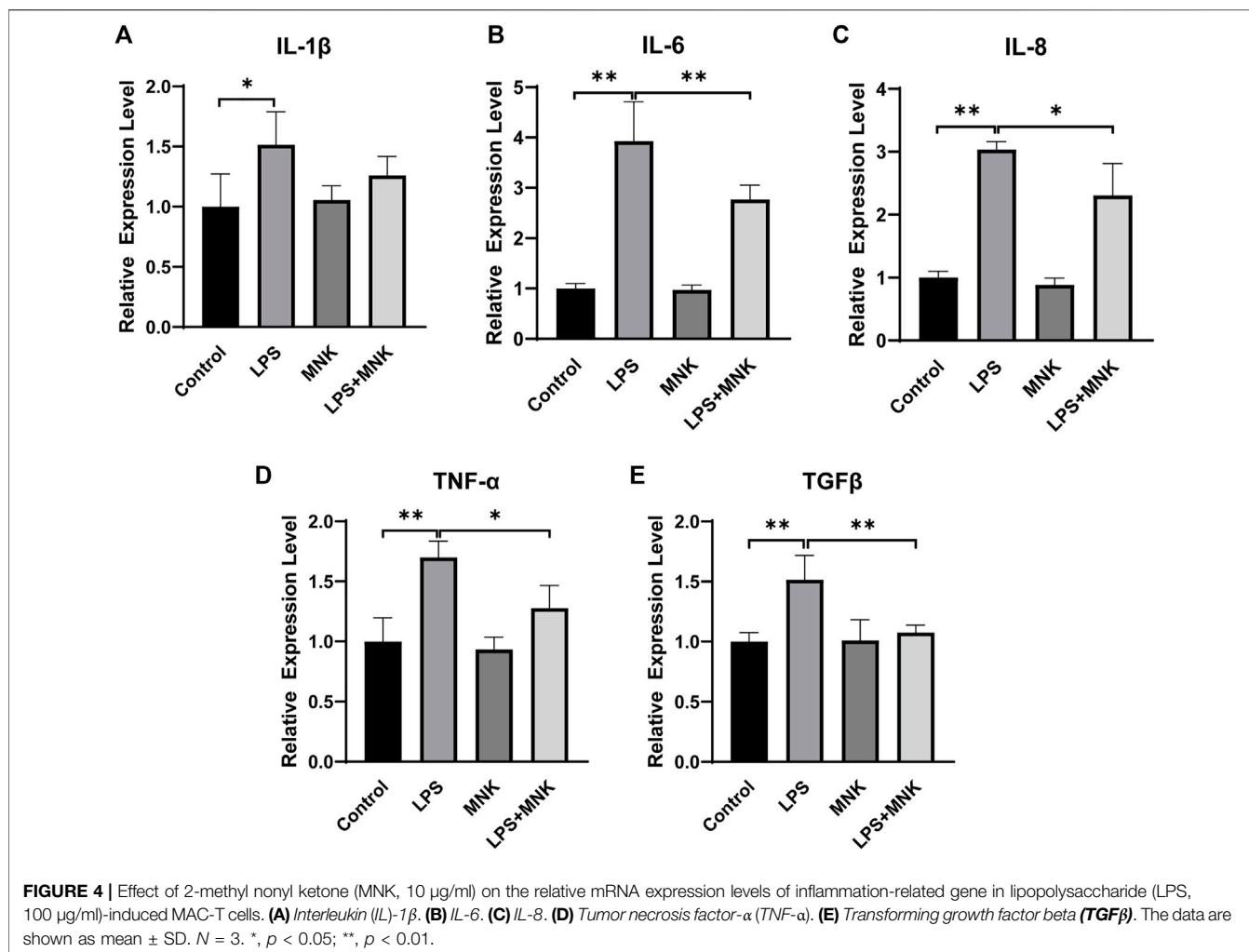
Effect of MNK on Nrf2 Signaling Pathway Induced by LPS in MAC-T Cells

The protective effect of MNK on LPS-induced MAC-T cell injury was investigated by Western blotting to determine whether it was related to the Nrf2 signaling pathway. The results showed that Nrf2 protein expression was significantly decreased in the LPS group compared to the control group. Nrf2 protein expression in the LPS + MNK groups was not significantly different from the control group. MNK reversed the reduction of Nrf2 protein expression induced by LPS (**Figure 7B**). The protein expression levels of p-AKT, AKT, p-ERK and ERK were further analyzed to determine whether the effect of MNK increasing the Nrf2 expression was related to the AKT and ERK pathways. As shown in **Figure 7C**, the p-AKT/AKT protein levels were significantly increased in the LPS plus

MNK group compared with the LPS group. Compared with the control group, the addition of MNK resulted in a significant increase in p-ERK/ERK. In addition, the mRNA expression level of *HO-1* was significantly increased in the LPS plus MNK groups compared to the LPS group.

DISCUSSION

Microbial infection is the main cause of mastitis (Graber and Bodmer, 2019). Pathogens invade the mammary gland and release macromolecules such as LPS to stimulate the immune response and cause mastitis (Cheng et al., 2019). However, antibiotics for mastitis cause antibiotic residues in milk, which damage human health (Anika et al., 2019). The development of more effective agents alternative to antibiotics for mastitis treatment is urgently needed. *H. cordata* has high nutritional and medicinal value and can be used in the treatment of inflammation (Shingnaisui et al., 2018) and has promising market development prospects as an alternative to antibiotics. However, the pharmacological effects and mechanisms for each component of *H. cordata* have not been fully investigated due to its complex composition. This is not conducive to its quality control and dose determination, which limits the further application



of *H. cordata*. MNK is the most abundant component of the volatile oil from the hydrodistillation extraction of *H. cordata* (Lou et al., 2019). And there have been some researches showing that MNK reduces adverse symptoms by inhibiting inflammatory responses in mice (Chen et al., 2014). However, the anti-inflammatory effect of MNK on bovine mammary epithelial cells and its molecular mechanism have not been investigated. LPS induces cell death by stimulating an increase in inflammatory cytokines and ROS (Qiu et al., 2019). In this study, the inflammatory model of mastitis in dairy cows was simulated using LPS-treated MAC-T cells to investigate the protective effect of adding MNK against LPS-induced cell damage. This work will contribute to the understanding of the anti-inflammatory effects of *H. cordata* and also provide a basis for the development of natural and effective drug candidates.

Bacterial infection of the mammary gland is accompanied by the release of endotoxins, proteoglycans and enzymes (Wang et al., 2021). This stimulates the immune response, leading to an increase in the number of somatic cells in the milk and the level of circulating endotoxin, which leads to an increase in the expression of pro-inflammatory cytokines and chemokines (Ashraf and Imran, 2020). IL-6, IL-8, and TNF- α are important pro-inflammatory cytokines involved in mastitis (Zhu et al., 2007; Imam et al., 2021; Liu et al.,

2021). In the present study, LPS significantly decreased the cell viability of MAC-T cells with a dose-dependent effect, indicating that LPS causes cell damage to MAC-T cells. Exogenous addition of MNK reversed the LPS-induced decrease in cell viability. This suggests that MNK has a protective effect on LPS-induced cell injury. Next, 100 $\mu\text{g/ml}$ LPS was selected to establish an inflammation model to simulate mastitis, and the expression levels of mRNA and protein of inflammation-related factors were examined. The results showed that MNK significantly inhibited LPS-induced upregulation of mRNA levels of IL-1 β , IL-6, IL-8, TNF- α , and TGF β and protein levels of STAT3, IL-6, TNF α , TNFR1 and TGF β . STAT3 is a downstream signaling regulatory molecule of IL-6 (Latourte et al., 2017). TNFR1 is a binding receptor for TNF- α , and TNF- α induces necroptosis in cells through TNFR1 (Amin et al., 2018). It implies that MNK acts cytoprotectively by reducing the production of pro-inflammatory cytokines and inhibiting their binding to receptors.

TLR4 is essential for LPS-stimulated inflammatory factor release (Bhattarai et al., 2018). LPS forms a dimeric complex with TLR4, which then activates downstream signaling pathways via MYD88 (Song et al., 2014). NF- κB plays a key role in promoting inflammatory cytokine expression (Chen et al.,

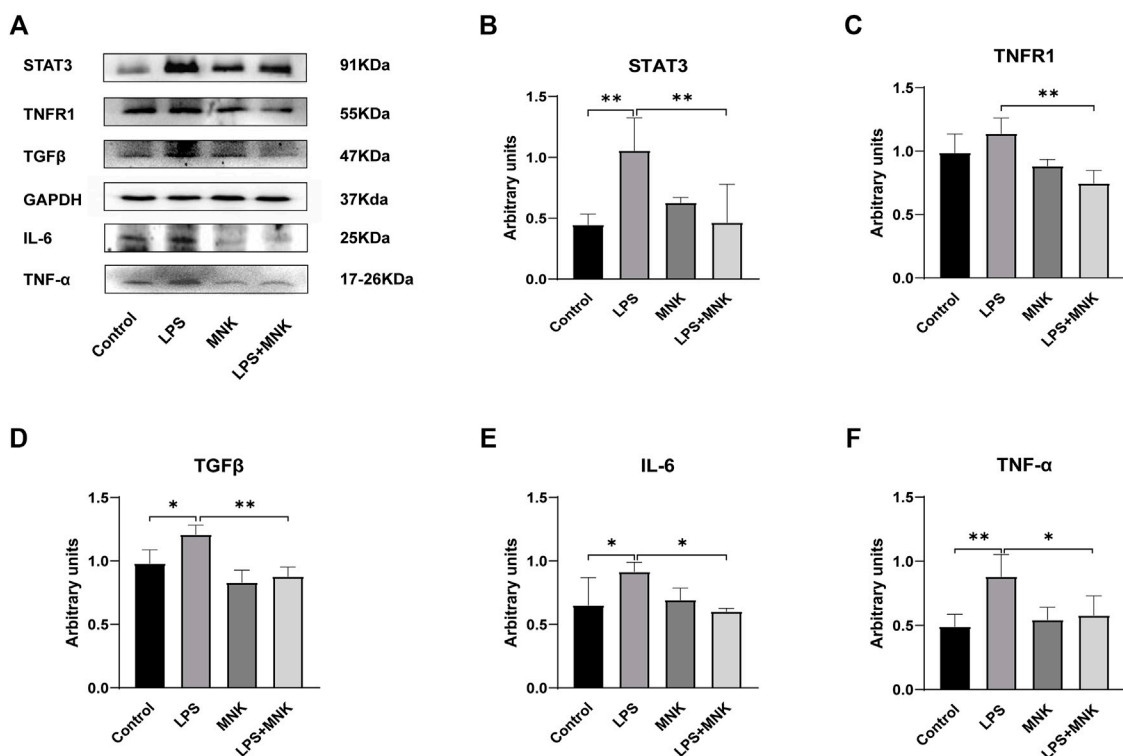


FIGURE 5 | Effect of 2-methyl nonyl ketone (MNK, 10 $\mu\text{g}/\text{ml}$) on the expression levels of inflammation-related proteins in lipopolysaccharide (LPS, 100 $\mu\text{g}/\text{ml}$)-induced MAC-T cells. **(A)** The expression of the inflammation-related protein was measured using Western blot. **(B)** Relative levels of signal transducer and activator of transcription (STAT3) were analyzed by grey scanning. **(C)** TNF-receptor-associated complex I (TNFR1). **(D)** Transforming growth factor beta (TGF β). **(E)** Interleukin (IL)-6. **(F)** Tumor necrosis factor- α (TNF- α). GAPDH was used as an internal reference for Western blotting analysis. The data are shown as mean \pm SD. $N = 3$. *, $p < 0.05$; **, $p < 0.01$.

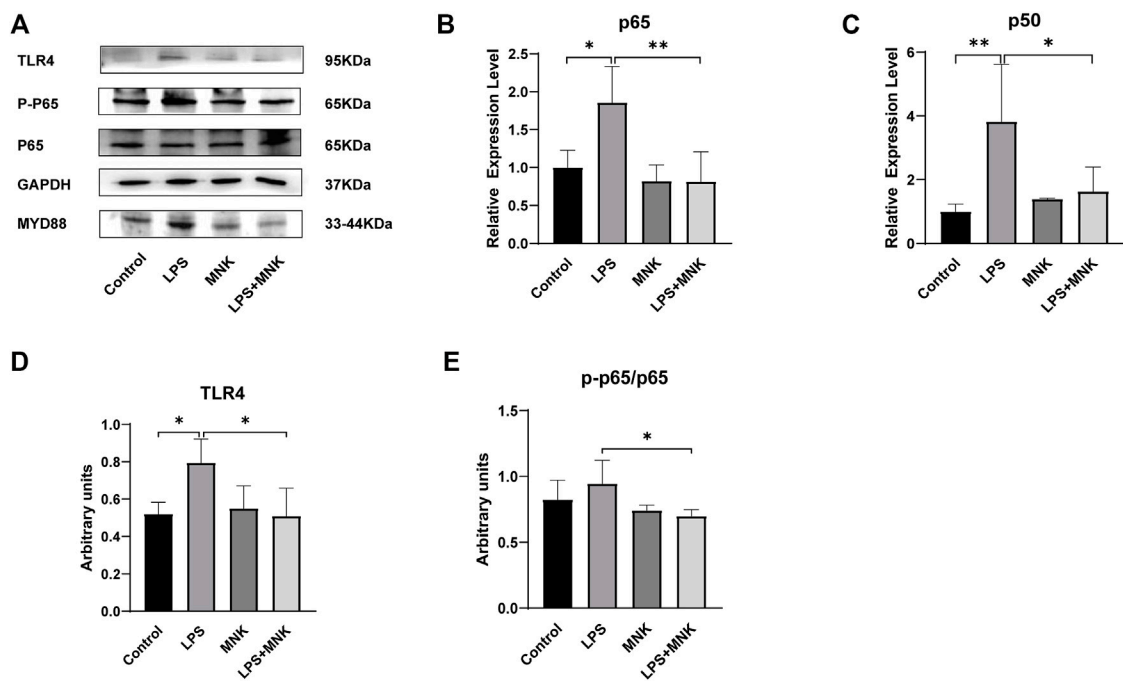


FIGURE 6 | The effects of 2-methyl nonyl ketone (MNK, 10 $\mu\text{g}/\text{ml}$) on the levels of Toll-like receptor 4-nuclear factor- κB (TLR4-NF- κB) signaling pathway induced by lipopolysaccharide (LPS, 100 $\mu\text{g}/\text{ml}$) in MAC-T cells. **(A)** The expression of the TLR4 signaling pathway was measured using Western blot. **(B)** p65. **(C)** p50. **(D)** TLR4. **(E)** p-p65/p65. GAPDH was used as an internal reference for Western blotting analysis. The data are shown as mean \pm SD. $N = 3$. *, $p < 0.05$; **, $p < 0.01$.

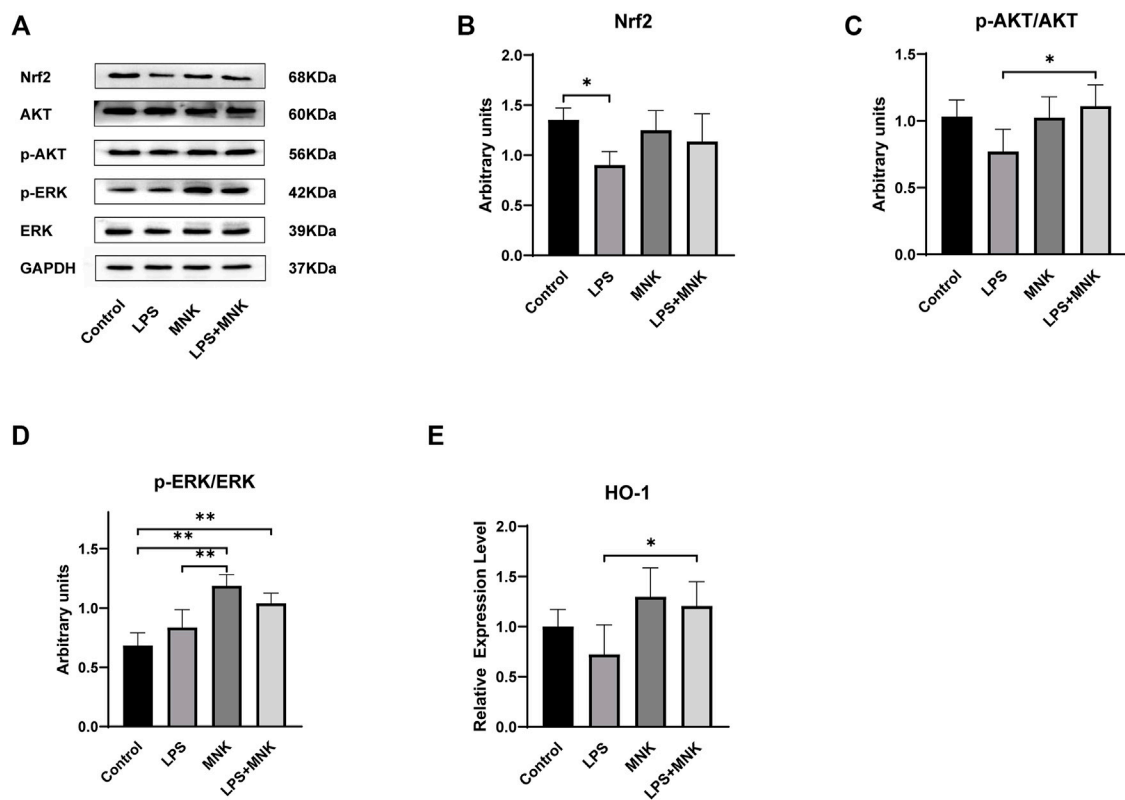


FIGURE 7 | The effects of 2-methyl nonyl ketone (MNK, 10 $\mu\text{g}/\text{ml}$) on the levels of Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway induced by lipopolysaccharide (LPS, 100 $\mu\text{g}/\text{ml}$) in MAC-T cells. **(A)** The expression of the Nrf2 signaling pathway was measured using Western blot. **(B)** Nrf2. **(C)** p-AKT/AKT. **(D)** p-extracellular signal-regulated kinase (ERK)/ERK. **(E)** The mRNA relative expression levels of *Heme Oxygenaseoxygenase-1* (*HO-1*). GAPDH was used as an internal reference for Western blotting analysis. The data are shown as mean \pm SD. $N = 3$. *, $p < 0.05$; **, $p < 0.01$.

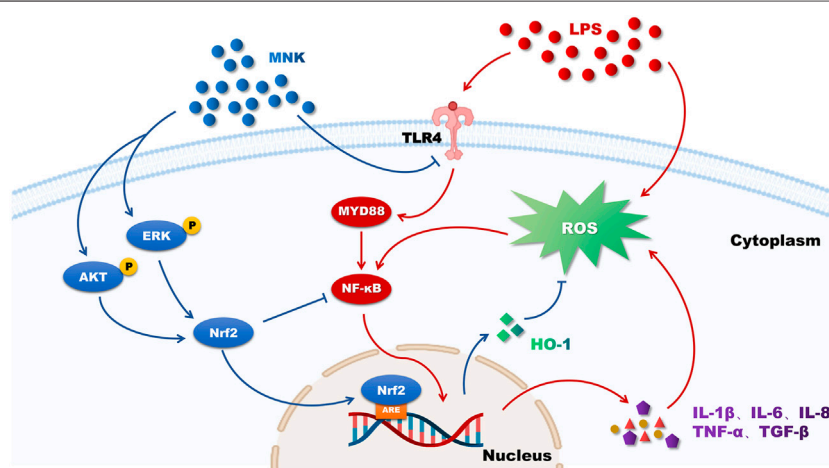


FIGURE 8 | The putative mechanism of 2-methyl nonyl ketone (MNK) attenuating the inflammatory response and oxidative stress of MAC-T cells induced by lipopolysaccharide (LPS). Red arrows indicate the effects of LPS on MAC-T cells and blue arrows indicate the protective effects of MNK.

2018). To further investigate the mechanism by which MNK exerts the anti-inflammatory effects, this study examined the effects of MNK on LPS-induced TLR4, MYD88, and NF- κ B

signaling pathways. In this study, MNK inhibited the expression of TLR4, MYD88 (**Supplementary Material S1**), NF- κ B and LPS-induced phosphorylation of p65. This

demonstrates that MNK has an anti-inflammatory effect in the LPS-induced mastitis model by inhibiting the TLR4-NF- κ B signaling pathway.

LPS disrupts the homeostasis of the redox system in bovine mammary epithelial cells, leading to increased levels of ROS and oxidative stress. Oxidative stress leads to mitochondrial dysfunction, abnormal protein synthesis, and induction of cellular autophagy to cause cellular damage (Ju et al., 2021). The results of this study showed that MNK pretreatment reduced the LPS-induced elevation of ROS levels. This indicates that MNK could reduce LPS-induced oxidative stress in MAC-T cells. Nrf2 is an important regulator that induces the expression of antioxidant enzymes (Zhou et al., 2021). Previous studies have shown that activation of AKT and ERK pathways can increase Nrf2 nuclear translocation thereby inhibiting the overproduction of ROS (Wang et al., 2019; Cao et al., 2021). The results of the present study are consistent with the findings that MNK activated AKT and ERK pathways and increased Nrf2 protein expression, which in turn promoted *HO-1* expression and attenuated LPS-induced oxidative stress. Excessive ROS triggers inflammation (Li et al., 2021). MNK may also inhibit the activation of NF- κ B pathway by reducing ROS levels through Nrf2 pathways, which in turn exerts anti-inflammatory effects.

In conclusion, MNK has a protective effect on LPS-induced mammary epithelial cell damage in cows (Figure 8). This suggests that MNK may serve as an effective candidate for the treatment of mastitis in dairy cows, but it needs to be verified by *in vivo* experiments in dairy cows. This work will provide a theoretical basis for the study of the pharmacodynamic mechanism of *H. cordata* and its application in the prevention and treatment work of mastitis in dairy cows.

CONCLUSION

In this study, MNK was found to have a protective effect on the inflammatory response and oxidative stress in mammary

epithelial cells of dairy cows. Pretreatment with MNK reduced the LPS-induced inflammatory response by inhibiting the TLR4-NF- κ B signaling pathway and pro-inflammatory factors receptor recognition. In addition, MNK may activate Nrf2 through AKT and ERK pathways, which in turn inhibits ROS production. This facilitates the protection of MAC-T cells from LPS-induced inflammatory response and oxidative damage.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

NW, CL, and XZ contributed to conception and design of the study. YZ performed the statistical analysis. NW wrote the first draft of the manuscript. DL, WB, YH, KL, YL, and LC wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.793475/full#supplementary-material>

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