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Protective effect of nerolidol on lipopolysaccharide-induced acute lung injury through the inhibition of NF-κB activation by the reduction of p38 MAPK and JNK phosphorylation

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ABSTRACT

Acute lung injury (ALI) is a severe syndrome, and there are no effective therapeutic appropriate medicines. Nerolidol, which exists in the essential oils of aromatic plants and flowers, exhibits antioxidative and anti-inflammatory activities. The present study evaluated the potential protective effect of nerolidol in ALI and the related mechanism in lipopolysaccharide (LPS)-treated mice. Here, nerolidol inhibited the LPS-induced neutrophil and other leukocyte infiltration of the alveolar space. LPS increased cytokines, chemokines, and adhesion molecules, and proinflammatory protein production was inhibited by nerolidol. LPS-induced phosphorylation of NF-κB p65, p38 MAPK, JNK, ERK were inhibited by nerolidol. The inhibitory concentration of nerolidol for the phosphorylation of NF-κB p65 and its upstream factors, p38 MAPK and JNK, was similar to the inflammatory responses of ALI. In conclusion, nerolidol is a potential protective agent in ALI via the inhibition of NF-κB activation and its upstream factors phosphorylation of p38 MAPK and JNK.

1. Introduction

Acute lung injury (ALI) is a severe disease consisting of acute inflammation caused by changes in the alveolar–capillary barrier permeability due to the overactivation of leukocytes (Hughes & Beasley, 2017; Johnson & Matthay, 2010). Neutrophils are the major type of leukocyte partipating in the pathogenesis of ALI (Grommes & Soehnlein, 2011). To simulate the clinical syndromes associated with ALI, intranasal administration of Gram-negative bacterial lipopolysaccharide (LPS) into mice is the most adopted approach (Matute-Bello, Frevert, & Martin, 2008). After intranasal administration of LPS, the innate immune system of the lung, containing alveolar macrophages and epithelial cells, activates its defence against the invasive pathogens (Johnson & Matthay, 2010). Chemokines, including macrophage inflammatory protein 2 (MIP-2), and cytokines, including interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α, are generated through the activation of the alveolar innate immune system (Ho et al., 2017; Lee et al., 2018). These chemokines and cytokines lead to neutrophil activation, which results in the generation of proinflammatory mediators, including nitrogen oxide (NO), reactive oxygen species, and prostaglandin (PG) (Grommes & Soehnlein, 2011). The activated neutrophils are recruited into the lungs through the disruption of the alveolar–capillary barrier induced by the proinflammatory mediators. The nuclear factor (NF)-κB pathway and its upstream factors, mitogen-activated protein kinases (MAPKs) including extracellular signal–regulated kinases (ERK), p38 MAPK, and c-Jun N-terminal kinases (JNK),

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play a major role in the regulation of chemokines, cytokines, and proinflammatory mediator expression in LPS-induced ALI (Shaukat et al., 2019).

To date, ALI and its corresponding severe-stage disease, acute respiratory distress syndrome (ARDS), have relatively high incidence and mortality worldwide (Johnson & Matthay, 2010). The incidence of ALI is approximately 200,000 patients each year in the United States. The mortality of patients with ALI is approximately 30%, but the mortality in older patients can be higher than 60% (Liu et al., 2007; Rubenfeld & Herridge, 2007; Toy et al., 2012). Except for mechanical ventilation, no appropriate therapeutic strategies reduce ALI-associated incidence or mortality (Schmidt, 2016). Nerolidol is an essential oil naturally occurring in aromatic plants and flowers, such as neroli, ginger, lavender, tea tree, and lemongrass (Azzi, Auezova, Danjou, Fourmentin, & Greige-Gerges, 2018; Pacifico et al., 2008). Nerolidol has several biological and pharmacological benefits including antioxidative, anti-inflammatory, antimicrobial, and antibacterial activities. It also enhances skin penetration and permeation (Chan, Tan, Chan, Lee, & Goh, 2016). Recent, we have purposed that nerolidol suppressed the inflammatory response during LPS-induced ALI via the modulation of antioxidant enzymes and the 5′ AMP-activated protein kinase (AMPK)/ nuclear factor erythroid 2–related factor (Nrf)-2/ heme oxygenase (HO)-1 pathway (Ni et al., 2019). However, nerolidol's role as an improving reagent in LPS-induced ALI via NFκB and MAPK pathway remains unclear. Therefore, we evaluated the protective effect of nerolidol on LPS-induced ALI mouse models and investigated the protective mechanisms.

2. Materials and methods

2.1. Reagents and assay kits

The Enzyme-linked immunosorbent assay (ELISA) kits for mouse TNFα, IL-1β, IL-6, MIP-2, ICAM (intercellular adhesion molecule)-1, and vascular cell adhesion molecule (VCAM)-2 were obtained from Cayman (Ann Arbor, MI, USA). The anti-iNOS, COX2, IκB, β-actin, phosoho(P)-p65, p65, ERK, P-ERK, JNK, P-JNK, P-p38 MAPK, p38 MAPK antibodies, and nerolidol were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradishperoxidas (HRP)-conjugated secondary antibodies used for the Western blots were obtained from Jackson ImmunoResearch Laboratories (Baltimore, MD, USA). LPS (*Escherichia coli* 0111:B4), dimethyl sulfoxide (DMSO) and other chemical agents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The final concentration of DMSO was less than < 0.2% (v/v).

2.2. Animals and experimental procedure

Male BALB/c mice, weighing 28–33 g and free of specific pathogens, were purchased from the National Laboratory Animal Centre (Taipei, Taiwan). All animal procedures in the present study were conducted in accordance with the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC Approval NO. 1848). The animals were divided randomly into six groups: a control group, an lipopolysaccharides (LPS) group (100 μg/20 μL), three nerolidol dosage (10, 30, and 100 μmol/kg) protective groups, and a dexamethasone (1 mg/kg) protective group. Control group mice were administered the vehicle intraperitoneally (IP) for 30 min before being administered 20 μL of saline intranasally. In the other groups, mice were administered the vehicle, nerolidol (10, 30, or 100 μmol/kg), or dexamethasone (1 mg/kg) for 30 min before being administered 20 μL of LPS intranasally. Mice were sacrificed 1 day after LPS administration, and the tissues and bronchoalveolar lavage fluid (BALF) were collected (Lee et al., 2018).

2.3. Cell counts and neutrophil counts in BALF

After the BALF was centrifuged at 1000*g* for 10 min, the resultant cell pellet was resuspended in phosphate-buffered saline (PBS). The total cell counts were measured using a haemocytometer. After cytospinning, the cells were stained using the modified Wright–Giemsa staining method. The percent of neutrophils was determined under a microscope. The neutrophil counts were calculated as total cell count × percentage of neutrophils (Lee et al., 2018).

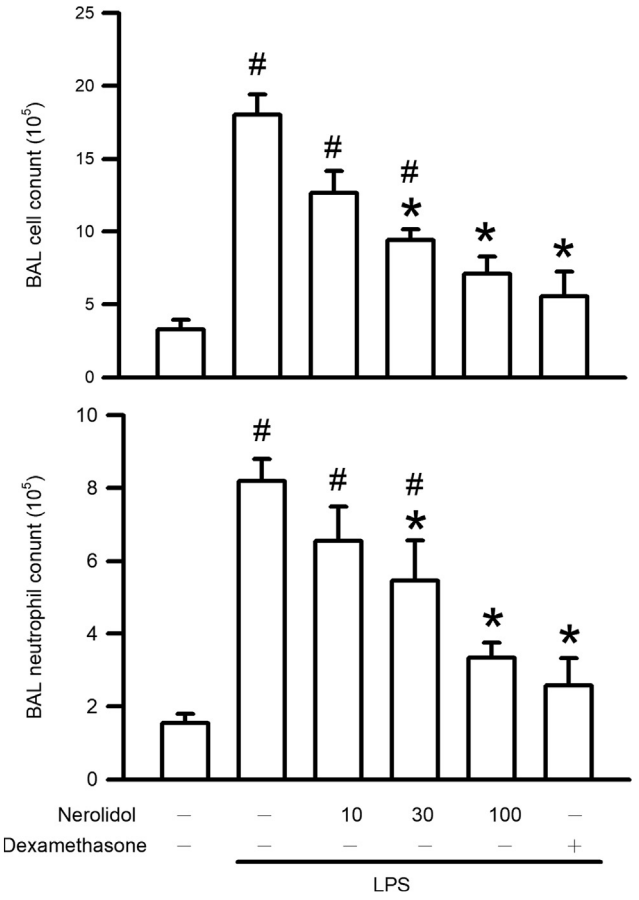

Fig. 1. Effects of nerolidol on LPS-induced total cells (A) and neutrophils infiltration (B) in ALI mice. Total cells and neutrophils infiltration determined by leukocyte counts in BALF. Values are expressed as mean ± S.D. (n = 4 in each group). # Represents significant difference between the indicated and control group; * between the indicated and LPS groups, $P < 0.05$.

2.4. Measurement of adhesion molecules and cytokines in BALF

After BALF centrifugation, the contents of adhesion molecules, including ICAM-1 and VCAM-2, and cytokines, including TNFα, IL-1β, IL-6, and MIP-2, in the supernatants of the BALF were measured using sandwich ELISA kits (Ho et al., 2017). The experimental procedures and quantification were conducted according to the manufacturer's instruction.

2.5. Western blot assay

The lungs were harvested and preserved in liquid nitrogen until homogenisation. The proteins isolated from the lungs were homogenised in tissue protein extraction solution, to which was added the proteinase and phosphatase inhibitor cocktail. Protein samples of 100 μg for each group were separated through sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were sealed and blocked with 5% skim milk (w/v) dissolved in PBS containing 0.1% Tween 20. The membranes were incubated with the

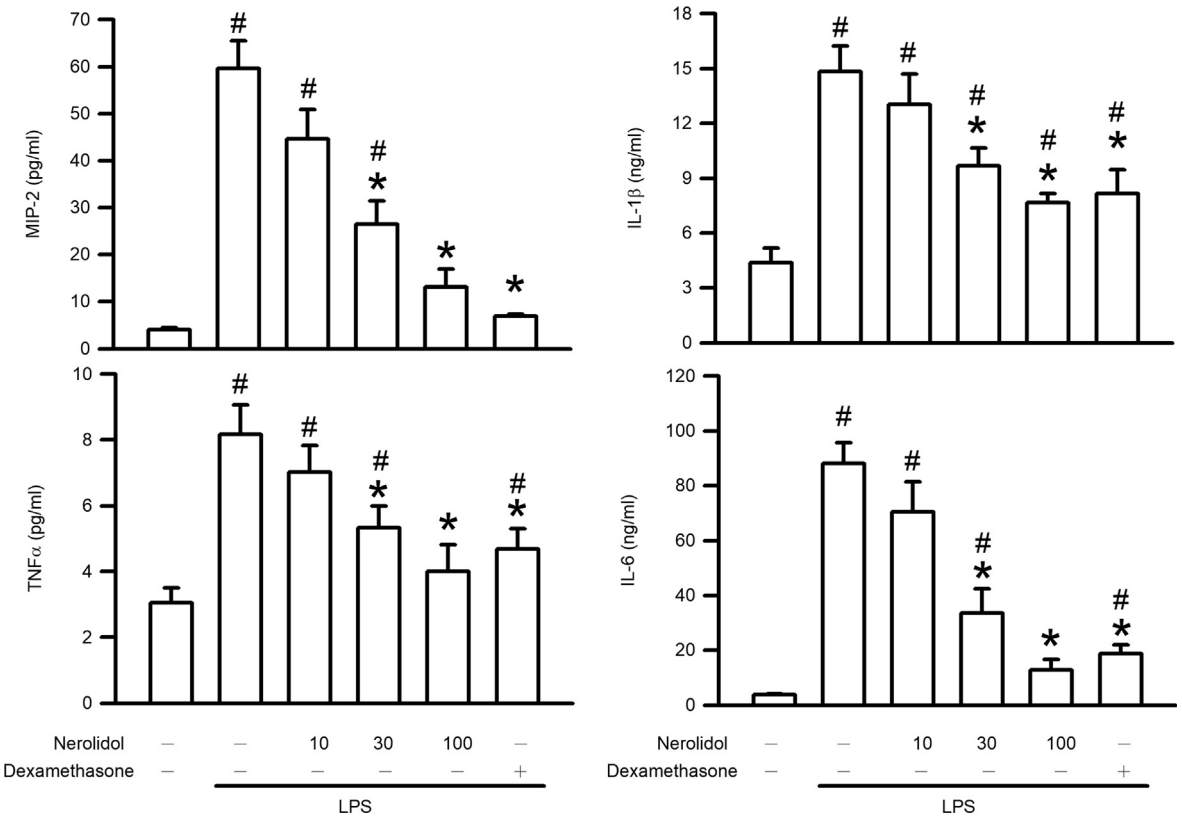

Fig. 2. Effects of nerolidol on LPS-induced secretion of chemokines and cytokines in ALI mice. Chemokine represented is MIP-2 (A). Cytokines represented are TNFα (B), IL-1β (C), and IL-6 (D). The level of chemokines and cytokines was determined by the ELISA assay. Values are expressed as mean ± S.D. (n = 4 in each group). # Represents significant difference between the indicated and control group; * between the indicated and LPS groups, $P < 0.05$.

respective primary antibodies overnight at 4 °C. The membranes were HRP-conjugated with secondary antibodies at room temperature for 1 h. Blots were developed with ECL western blotting reagents. Finally, the signals were determined quantitatively with the image analysis system (Infinity CX 5, Vilber Lourmat, France) (Ho et al., 2017).

2.6. Statistical analysis

All data are expressed as mean values ± standard deviation (S.D.). Differences between the groups were analysed through one-way analysis of variance followed by the Bonferroni *t* test in SPSS 14.0 statistical software. *P* values less than 0.05 are considered statistically significant.

3. Results

3.1. Effects of nerolidol on LPS-induced neutrophil infiltration in ALI models

To investigate whether LPS-induced ALI was ameliorated by nerolidol, the mice were preadministered nerolidol at various concentrations for 30 min before LPS administration. The critical histopathological feature of ALI is neutrophil infiltration. The haemocytometer measurement and Wright–Giemsa staining revealed that the total cell counts and neutrophil counts increased in the BALF of LPS-challenged mice. Pretreatment with nerolidol reduced the LPS-induced total cell and neutrophil infiltration in a dose-dependent manner starting at 30 μmol/kg ($P < 0.05$, Fig. 1).

3.2. Effects of nerolidol on the LPS-induced secretion of chemokines and cytokines in ALI models

Cytokines and chemokines play major roles in neutrophil recruitment into the lung during intranasal administration of LPS (Ho et al., 2017; Li et al., 2009; Lee et al., 2018). The effect of nerolidol on the secretion of chemokines, including MIP-2, and cytokines, such as TNFα, IL-1β, and IL-6, in BALF, was analysed through ELISA. The levels of chemokines and cytokines were significantly higher compared with the control group ($P < 0.05$) after the administration of LPS for 24 h. Pretreatment with nerolidol reduced the LPS-induced generation of chemokines and cytokines in a dose-dependent manner starting at 30 μmol/kg ($P < 0.05$, Fig. 2).

3.3. Effects of nerolidol on the LPS-induced expression of adhesion molecules in ALI models

The expression of adhesion molecules on the cellular surface of the endothelium and leukocytes mediates neutrophil recruitment (Lee et al., 2018). The effect of nerolidol on the expression of adhesion molecules, including VCAM-1 and ICAM-1, in BALF was analysed through ELISA. The levels of VCAM-1 and ICAM-1 were significantly higher compared with the control group ($P < 0.05$) after administration of LPS for 24 h. Pretreatment with nerolidol reduced the LPS-induced generation of VCAM-1 and ICAM-1 in a dose-dependent manner starting at 30 μmol/kg ($P < 0.05$, Fig. 3).

3.4. Effects of nerolidol on the LPS-induced expression of iNOS and COX2 in ALI models

Proinflammatory proteins, including iNOS and COX2, from activated leukocytes result in tissue damage in LPS-induced ALI (Lee et al., 2018). As illustrated in Fig. 4, the expression of proinflammatory proteins, iNOS and COX2, was significantly higher in the LPS-treated mice compared with the control group. Dose-dependent inhibition was observed in the nerolidol-treated groups. The significant inhibitory effect

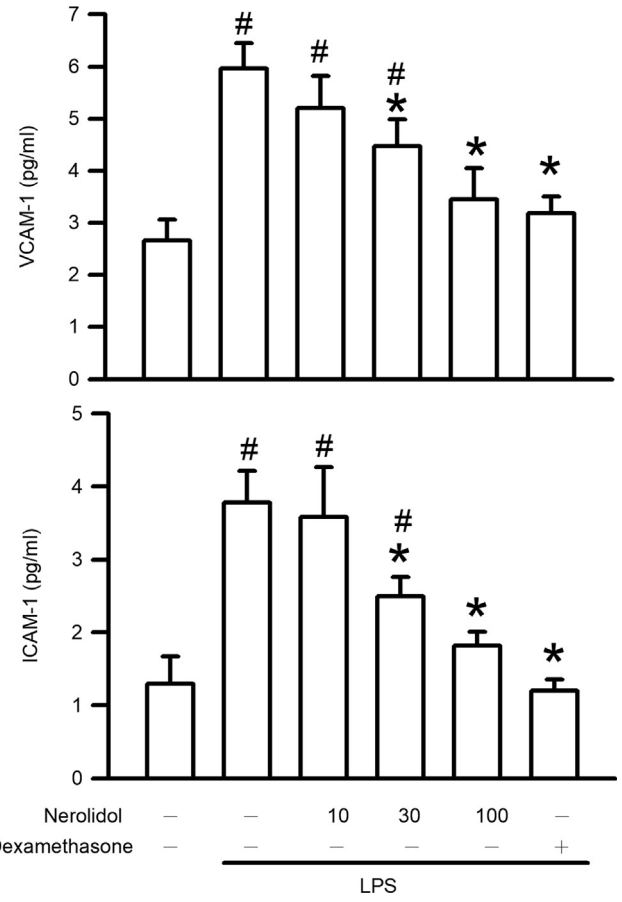

Fig. 3. Effects of nerolidol on LPS-induced expression of adhesion molecules in ALI mice. Adhesion molecules represented are VCAM-1 (A) and ICAM-1 (B). The level of adhesion molecules was determined by the ELISA assay. Values are expressed as mean ± S.D. (n = 4 in each group). # Represents significant difference between the indicated and control group; * between the indicated and LPS groups, $P < 0.05$.

began at 30 μmol/kg ($P < 0.05$, Fig. 4).

3.5. Effects of nerolidol on the LPS-induced activation of the NF-κB pathway in ALI models

The NF-κB pathway participates in the secretion of chemokines and cytokines, expression of adhesion molecules, and generation of proinflammatory proteins in LPS-induced ALI (Lee et al., 2018). The phosphorylation of IκB, degradation of IκB, and the phosphorylation of NF-κB p65 were significantly higher in the mice that were administered LPS for 24 h compared with the control group ($P < 0.05$). Pretreatment with nerolidol reduced the LPS-induced phosphorylation of IκB, degradation of IκB, and phosphorylation of NF-κB p65 in a dose-dependent manner starting at 30 μmol/kg ($P < 0.05$, Fig. 5).

3.6. Effects of nerolidol on the LPS-induced phosphorylation of MAPKs in ALI models

The phosphorylation of MAPKs, including ERK, JNK, and p38 MAPK, plays a key role in the LPS-induced activation of NF-κB in ALI (Shaukat et al., 2019). The phosphorylation of MAPKs was significantly higher in the mice that were administered LPS for 24 h compared with the control group ($P < 0.05$). Pretreatment with nerolidol reduced the LPS-induced phosphorylation of JNK and p38 MAPK in a dose-dependent manner starting at 30 μmol/kg ($P < 0.05$). However, the LPS-induced phosphorylation of ERK was significantly inhibited by

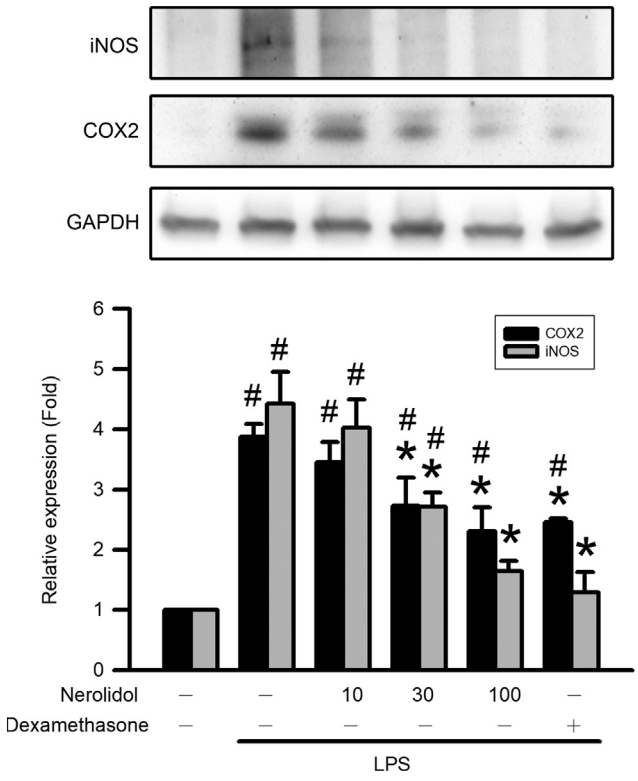

Fig. 4. Effects of nerolidol on the LPS-induced expression of iNOS and COX2 in ALI mice. The lung lysates were analyzed by western blotting. The fold of iNOS and COX2 expression between the treated and control groups was calculated. Values are expressed as mean ± S.D. (n = 3 in each group). #Represents significant difference between the indicated and control group; *between the indicated and LPS groups, $p < 0.05$.

nerolidol at 100 μmol/kg ($P < 0.05$, Fig. 6).

4. Discussion

When patients with ALI and ARDS are administered anti-inflammatory drugs, serious adverse events occur, and high medical cost burden. The beneficial compounds found in plants, traditional medicines, and phytopharmaceuticals have been a considerable source for the development of potential therapeutic reagents (Favarin, de Oliveira, de Oliveira, & Rogerio, 2013). Nerolidol, also called peruviol, is a sesquiterpene alcohol extracted from the natural essential oil of fragrant plants such as Aframomum pruinosum and *Cinnamomum chartophyllum* (Botelho et al., 2007; Lima et al., 2012; Marqueset al., 2011; Pacifico et al., 2008; Parreira et al., 2010; Tan et al., 2015; Zhou et al., 2018). Nerolidol has long been as a common fragrance ingredient and as a flavouring agent in pharmaceutical formulations and foods. Previous studies have suggested that nerolidol has antioxidant, anti-inflammatory, antimicrobial, and antibacterial bioactivities (Chan et al., 2016). Essential oil from *Cinnamomum chartophyllum* and lemongrass is known as an aromatic or volatile oil providing a protective effect on the respiratory system (Jiang et al., 2017; Zhou et al., 2018). Therefore, we hypothesised that nerolidol could improve the inflammatory response in ALI induced by LPS.

Neutrophil recruitment into the lungs plays a key role in the pathogenesis of ALI (Grommes & Soehnlein, 2011). In patients with ALI and ARDS, the amount of neutrophil infiltration into the lungs and alveolar space is related to the severity and healing ALI and ARDS (Grommes & Soehnlein, 2011). In animal experiments, ALI induced by the administration of LPS through the respiratory tract results in similar pathological features to those observed in ALI patients, especially syndromes such as neutrophilic inflammatory response and

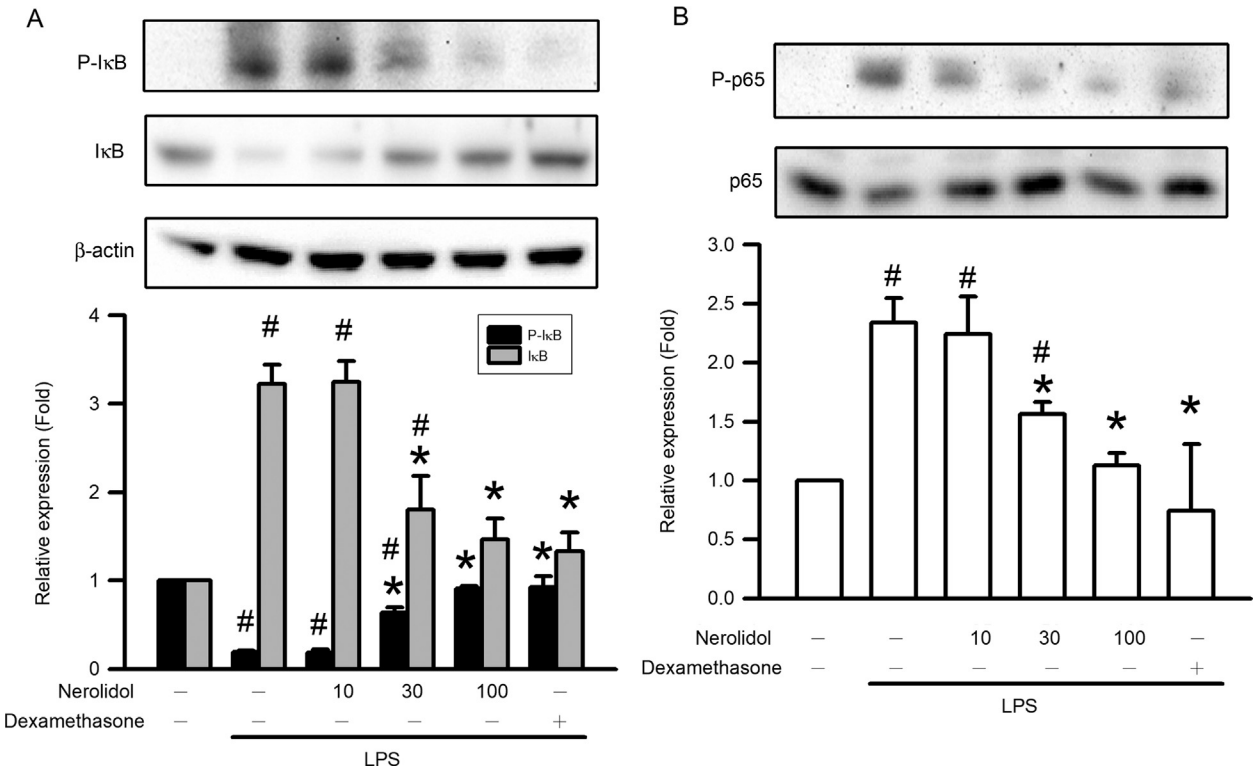

Fig. 5. Effects of nerolidol on the LPS-induced activation of the NF-κB pathway in ALI mice. The lung lysates were analyzed by western blotting. The fold of (A) IκB degradation and IκB phosphorylation, (B) p65 phosphorylation between the treated and control groups was calculated. Values are expressed as mean ± S.D. (n = 3 in each group). #Represents significant difference between the indicated and control group; *between the indicated and LPS groups, $p < 0.05$.

intrapulmonary cytokines (Matute-Bello et al., 2008). The severity of ALI was reduced after LPS administration in neutrophil-depleted mice (Abraham, Carmody, Shenkar, & Arcaroli, 2000). We also observed that the total cell and neutrophil infiltration of the alveolar space increased after LPS administration in mice with ALI. Neutrophil migration was also inhibited by the essential oil of Peperomia serpens, which contains nerolidol, in mice IP injected with carrageenan and dextran (Pinheiro et al., 2011). The results of the present study demonstrated that neutrophil and total cell infiltration of the alveolar space was reduced by nerolidol in LPS-induced ALI mouse models. These results indicate that nerolidol ameliorated the neutrophil infiltration induced by LPS in mice with ALI.

The recruitment of neutrophils into the lung through chemotaxis and migration are induced by the expression of chemokines and cytokines during LPS-induced ALI (Ho et al., 2017; Lee et al., 2018). IL-1β, TNFα, and IL-6 are the bioactive cytokines in the alveolar space in patients with ALI (Butt, Kurdowska, & Allen, 2016; Olman et al., 2002) The permeability of the alveolar–capillary barrier, secretion of proinflammatory cytokines and chemokines, and priming and emigration of neutrophils are increased by IL-1β, TNFα, and IL-6 (Basran et al., 2013; Butt et al., 2016; Snelgrove, & Lloyd, 2017; Weifeng et al., 2016). MIP-2 is the chemokine generated by lung microvascular endothelial cells, alveolar macrophages, and epithelial cells in ALI (Butt et al., 2016). Neutrophil mobilisation is stimulated by MIP-2 (Burdon, Martin, & Rankin, 2005). LPS-induced expression of IL-1β and TNFα was inhibited by nerolidol in the kidneys of rats with acute kidney injury, NRK-52E proximal tubular cells of rats, and peritoneal macrophages (Fonsêca et al., 2016; Zhang et al., 2017). Nerolidol inhibits rotenone, which increases the levels of IL-1β, IL-6, and TNF-α in rat brains (Javed, Azimullah, Abul Khair, Ojha, & Haque, 2016). We discovered upregulation of chemokine and cytokine expression in LPS-treated mice. Nerolidol reduced the level of MIP-2, IL-1β, IL-6, and TNF-α in BALF in a concentration-dependent manner. Based on these results, we suggest that the reduction in neutrophil infiltration by nerolidol was caused by the inhibition of MIP-2, IL-1β, IL-6, and TNF-α secretion.

The expression of adhesion molecules VCAM-1 and ICAM-1 on lung microvascular endothelial cells and neutrophils, respectively, plays a major role in neutrophil infiltration into the lungs in LPS-induced ALI (Lee et al., 2018). The proinflammatory mediators NO and PGE2 generated by iNOS and COX2, respectively, participated in the pathogenesis of ALI induced by LPS (Lee et al., 2018). Nerolidol exists in the essential oil of *P. serpens* and reduces the migration of neutrophils and adhesion of leukocytes in mice IP injected with carrageenan and dextran (Pinheiro et al., 2011). Nerolidol inhibits the rotenone-induced expression of iNOS and COX2 in rat brains (Javed et al., 2016). The LPS-induced expression of VCAM-1, ICAM-1, iNOS, and COX2 was reduced by nerolidol in the current study. The protective effect of nerolidol was involved in the reduced expression of adhesion molecules and proinflammatory mediators.

The inducible transcription factor NF-κB has been demonstrated to regulate the various signalling pathways of proinflammatory responses, including cytokine and chemokine secretion, adhesion molecule expression, and proinflammatory mediator generation (Hoesel & Schmid, 2013; Liu, Zhang, Joo, & Sun, 2017). Moreover, p65, also named RelA, is the amount component of NF-κB and binds to NF-κB DNA-enhancer sequences for the transcription of target genes (Hoesel & Schmid, 2013; Liu et al., 2017). In the resting state, p65 is located in the cytosol and bound to its inhibitor, IκB. After challenger with LPS, the phosphorylation of p65 is induced by the ubiquitination and degradation of IκB (Hoesel & Schmid, 2013; Liu et al., 2017). The main upstream factor of NF-κB activation in mice with LPS-induced ALI, including NF-κB phosphorylation and IκB degradation, is the phosphorylation of the MAPK family, including ERKs, p38 MAPK, and JNKs (Shaukat et al., 2019). The phosphorylation of NF-κB p65 was reduced by nerolidol in rats with LPS-induced acute kidney injury and in NRK-52E cells (Zhang et al., 2017). The essential oil of *Lindera erythrocarpa*, whose main compound is nerolidol, attenuates IκB degradation and NF-κB phosphorylation through the phosphorylation of the MAPK family in LPS-

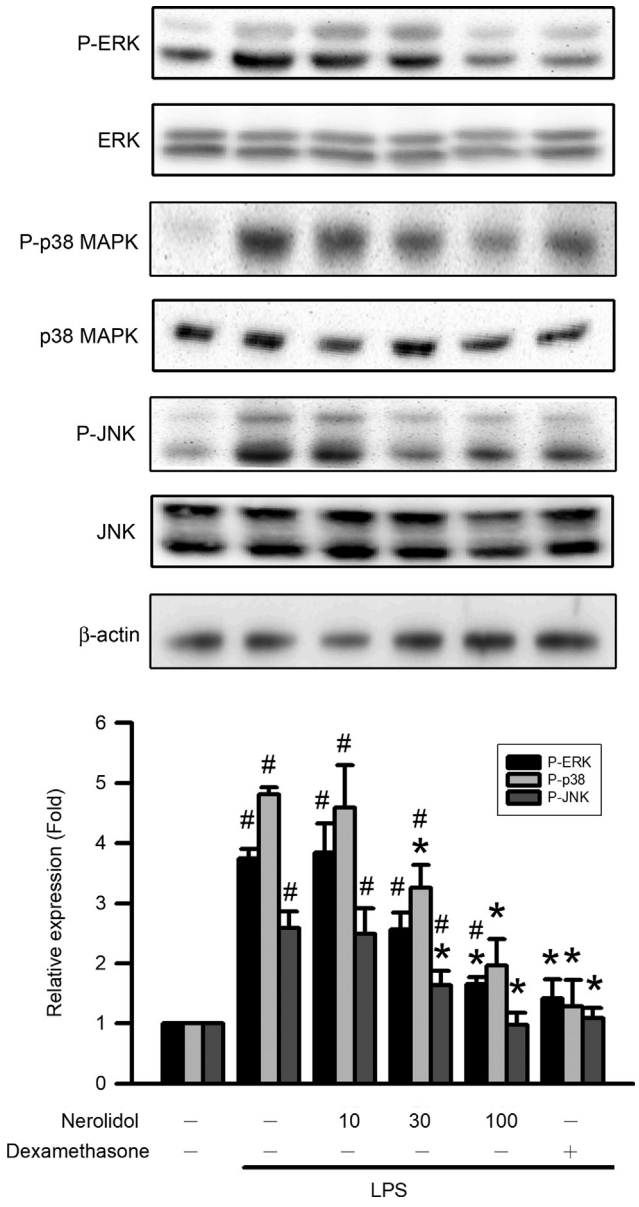

Fig. 6. Effects of nerolidol on the LPS-induced phosphorylation of MAPKs in ALI mice. The lung lysates were analyzed by western blotting. The fold of ERK, p38 MAPK, JNK phosphorylation between the treated and control groups was calculated. Values are expressed as mean ± S.D. (n = 3 in each group). #Represents significant difference between the indicated and control group; *between the indicated and LPS groups, $p < 0.05$.

activated RAW264.7 macrophages (Ko et al., 2017). In the present study, the activation of NF-κB, including p65 phosphorylation and IκB degradation, was induced by LPS administration and in turn significantly reduced by nerolidol at 30 μmol/kg in mice with ALI. The phosphorylation of p38 MAPK and JNKs was also inhibited significantly after pretreatment with nerolidol at 30 μmol/kg. However, the phosphorylation of ERKs was significantly inhibited by nerolidol only at 100 μmol/kg. The parallel trends of the activation of NF-κB and phosphorylation of p38 MAPK and JNKs were inhibited by nerolidol. According to these results, we propose that the activation of NF-κB was reduced by nerolidol through a primary regulated molecular pathway which is the inhibition of the phosphorylation of p38 MAPK and JNKs in LPS-induced ALI.

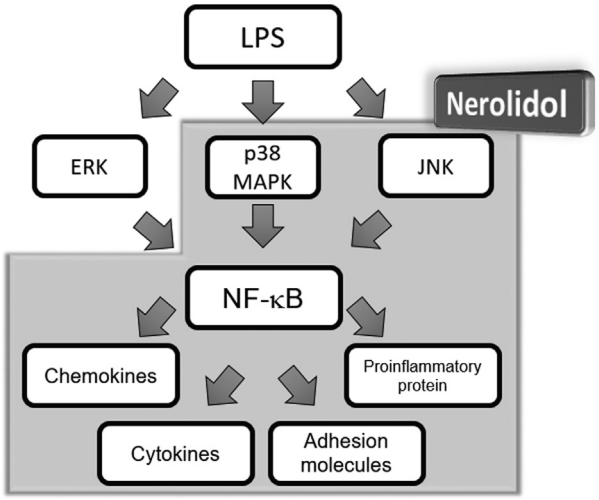

Fig. 7. Scheme of the mechanisms in the protective effect of nerolidol on LPS-induced ALI through the inhibition of NF-κB activation by the reduction of p38 MAPK and JNK phosphorylation. The regulatory molecules in the shadow area were inhibited by nerolidol.

5. Conclusion

In summary, we observed that nerolidol significantly suppressed neutrophil and leukocyte infiltration by inhibiting proinflammatory responses, such as the (1) generation of chemokines, including MIP-2, and cytokines, including TNFα, IL-1β, and IL-6; (2) expression of adhesion molecules, including VCAM-1 and ICAM-1; and (3) expression of proinflammatory proteins, including iNOS and COX2, in LPS-induced ALI mouse models. The mechanisms involved in the protective effect of nerolidol were the downregulation of the activation of NFκB and phosphorylation of p38 MAPK and JNKs. These results suggest the molecular mechanism of nerolidol acting as a protective reagent in LPS-induced ALI through the reversal of the NF-κB activation through the phosphorylation of p38 MAPK and JNKs (Fig. 7). The mechanisms for the anti-inflammatory effects of dexamethasone contain immunosuppression, downregulation of proinflammatory mediator production and transcription factors activation (Gorgani et al., 2011; Warcoin, Baudouin, Gard, & Brignole-Baudouin, 2016; Xian et al., 2019). Herein, generation of chemokines and cytotkines, expression of adhesion molecules and proinflammatory proteins, activation of NFκB and MAPK were reduced by dexamethasone in LPS-induced ALI mice. However, the adverse effects of dexamethasone have too much to clinically used. Therefore, it is expected to use the nerolidol instead of dexamethasone.

6. Ethics statements

All animal procedures in the present study were conducted in accordance with the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC Approval NO. 1848).

CRediT authorship contribution statement

Shih-Pin Chen: Conceptualization, Methodology, Writing - original draft, Data curation, Funding acquisition. **Chun-Hung Su:** Software, Writing - original draft, Visualization. **Rosa Huang-Liu:** Methodology, Software, Writing - original draft. **Min-Wei Lee:** Methodology, Software, Visualization. **Chen-Yu Chiang:** Methodology, Writing - original draft. **Wen-Ying Chen:** Methodology, Software, Visualization. **Chun-Jung Chen:** Methodology, Software, Writing - original draft. **Sheng-Wen Wu:** Conceptualization, Data curation, Visualization, Writing - review & editing, Project administration, Funding acquisition.

Yu-Hsiang Kuan: Conceptualization, Data curation, Writing - review & editing, Project administration, Funding acquisition.

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.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103943.

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