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Article

Ginsenoside Rh2 Ameliorates Lipopolysaccharide Induced Acute Lung Injury by Regulating the TLR4/ PI3K/Akt/Mtor, Raf-1/MEK/ERK and Keap1/Nrf2/HO-1 Signaling Pathways in Mice

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Abstract: The anti-inflammatory effect of ginsenoside Rh2 (GRh2) is one of the most important ginsenosides. The purpose of this study is to identify the anti-inflammatory and antioxidant effects of GRh2 after LPS challenge lung injury animal model. GRh2 reduced LPS-induced NO, TNF-α, IL-1β, IL-4, IL-6 and IL-10 productions in lung tissues. GRh2 treatment decreased the histological alterations in the lung tissues and BALF protein content and total cells number also diminished in LPS-induced lung injury mice. Moreover, GRh2 blocked iNOS, COX-2, the phosphorylation of IκB-α, ERK, JNK, p38, Raf-1 and MEK protein expression which is corresponded to the growth of HO-1, Nrf-2, catalase, SOD and GPx expressions in LPS-induce lung injury. An experimental study has suggested that GRh2 has provided with anti-inflammatory effects in *vivo*, and its potential therapeutic efficacy in major anterior segment lung diseases.

Keywords: Ginsenoside Rh2; Lipopolysaccharide; Acute lung injury; MEK; Nrf-2;

1. Introduction

Acute lung injury (ALI) continues to produce the high mortality rate and mortality that it seems to be related to the shock, sepsis, and ischemia reperfusion, aspiration of gastric contents, major trauma and acute pancreatitis [1]. ALI induced either by hypoxic and ischemic stresses or bacterial endotoxin such as lipopolysaccharide (LPS), is occur with the stability of redox states can cause DNA damage, oxidative protein and lipid [2]. The main features of ALI are the leakage of plasma proteins into alveolar space, accumulation of inflamed cells, pulmonary interstitial edema, and destruction of epithelial barrier integrity [3, 4]. Activation of macrophage participate inflammatory responses in LPS inducement by releasing pro-inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6], anti-inflammatory cytokines (IL-4 and IL-10) and pro-inflammatory mediator (NO) that relating to the sites of tissue injury in immune cell recruitment [5]. Toll-like receptor 4 (TLR4) is a transmembrane protein that belongs to the pattern recognition receptor for LPS from gram-positive bacteria and TLR4 activates the mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) to induce the production of inflammatory mediators [6]. TLR4 is a potential therapeutic marker to reduce inflammatory responses in ALI. Moreover, phosphatidylinositol-3 kinase (PI3K)/ protein kinase B (Akt)/ mechanistic target of rapamycin (mTOR) signaling pathway are an important intracellular mediator, which is critical for

the regulation of cell survival and proliferation [6, 7]. The PI3K/Akt/mTOR signal pathway has been implicated in the inflammatory disease, and its inhibition has become a useful therapy. In addition, oxidative stress signaling is involving in modulating LPS-induced inflammatory reaction that regulates through transcription factors NF-E2-related factor 2 (Nrf2) activation. Heme oxygenase-1 (HO-1) induction by Nrf2 protects against the cytotoxicity of various oxidative stresses and inflammatory response that is a stress-regulated protein through the Keap1/Nrf2/HO-1 pathway [7]. The Raf/MAPK kinase (MEK)/ERK signaling cascade has been best characterized previously in response to cell growth, proliferation, and survival [8]. The Raf/MEK/ERK signal pathway is detected after LPS-induced ALI; however, the effect of modulating the pathway in vivo is unknown.

The main active constituents of ginseng (the root of Panax ginseng Meyer; Korean ginseng) are ginsenosides that are getting significant attention in the human health in traditional medicine, such as anti-inflammatory, antioxidant, antidiabetic, antitumor, immunological regulation, and slow the aging process [9, 10].

Ginsenoside Rh2 (GRh2) with a dammarane skeleton that is a rare ginsenoside. More specifically, GRh2 only presents in red ginseng that supports anti-inflammatory, antitumor effects, improving memory and liver functions [11]. GRh2 has anti-tumor activity against leukemia, prostate cancer, pancreatic cancer and glioblastoma [12]. GRh2 also has reduced the allergy, improved atopic, and contact dermatitis by inhibiting the NF-κB activation, p38 MAPK phosphorylation and inflammatory cytokines [13, 14].

Several signal transduction pathways have been suggested to explain the activation of inflammatory mediators after LPS-induced ALI. Therefore, we hypothesized that TLR4/ PI3K/Akt/mTOR, Keap1/Nrf2/HO-1 and Raf-1/MEK/ERK signaling pathway is involved the inflammatory mechanisms of GRh2 after LPS challenge ALI. Thus, the purpose of this study finds out the anti-inflammatory and antioxidant effects of GRh2. our results suggest that GRh2 is a good choice to develop dietary supplements for preventing acute lung injury and inhibit inflammation.

2. Experimental

2.1. Reagents

GRh2 (Fig. 1A, purity > 98%), was kindly supplied by Professor Yuan-Shiun Chang. All reagents and solvents were acquired by Sigma-Aldrich (St Louis, MO, USA). Assay kits for the determination of mouse TNF-α, IL-1β, IL-4, IL-6, and IL-10 were received from Biosource International Inc. (Camarillo, CA, USA). Primary Antibodies were acquired from Abcam (Cambridge, England).

2.2. Animals

Male ICR (weighing 20-25 g) mice were purchased from the BioLASCO Taiwan Co., Ltd.. All animal procedures were conducted in accordance with the animal management committee of the China medical University (IACUC approval number: 104-93-N). Every effort is made to minimizing suffering the use of animals and reduces the number of animals used.

2.3. Experimental design

Mice were randomly selected and divided into the following six groups: control, LPS only, GRh2 (5, 10, and 20 mg/Kg) + LPS, and Dex (10 mg/kg; a positive drug) + LPS treatment groups (n = 6 in each group). ALI was induced by intratracheal instillation of LPS (5 mg/kg; 50 μL in sterile saline), then GRh2 and Dex was injected intraperitoneally 1h prior to LPS administration. The animals were sacrificed 6 h later and the sample was collected [6].

2.4. BALF collection and cell count

The BALF was collected from each individual mouse by lavaging the lung with normal saline three times and supernatants were then collected for later analysis by ELISA and protein study. And re-suspend the pellets in saline for total cell counts using a hemocytometer. The sediment was re-

suspended for the determinate total number of cells and protein content. Total cell number was acquired by hemocytometer.

2.5. Nitrites assay

Determination of nitrite level in BALF was measured by the Griess reagent [7]. Briefly, add the equal volume of Griess reagent and the BALF solution (1:1) was mixed. After 10 min of incubation the absorbance of supernatants was measured by a microplate photometer plate reader at 540 nm

2.6. Histopathological analysis

The lobe of the right lung was excised for histopathological analysis. The lung slices were fixed in 4% paraformaldehyde and dehydrated from water through a standard graded alcohol and embedding in paraffin. Histologic specimen of lung tissue stained with hematoxylin and eosin (H&E) stain and observed with a light microscope. The severity of lung injury scores from one to five depends on the degree of inflammatory infiltration, neutrophils and disseminated, which ranges from 0 to 5. A score of 0 expressed normality; 1 expressed minimal (<1%); 2 expressed slight (1-25%); 3 expressed moderate (26-50%); 4 expressed moderate/severe (51-75 **%**); 5 expressed severe/high (76-100**%**) [4].

2.7. Cytokine assay

BALF analyzed with commercially colorimetric test kit (Biosource International Inc., Camarillo, CA) to quantify TNF-α, IL-1β, IL-4, IL-6 and IL-10 production. The absorbance at 450 nm was measured using a microplate reader.

2.8. Lung wet to dry (W/D) weight ratio

Lung tissues from left lobes were collected to calculate tissue edema. The fresh lung was weighed and dried at 80°C in an oven for at least 24 h and reweighed when it was dry.

2.9. Myeloperoxidase activity

Lungs tissues were homogenized in sterile saline. The homogenate was centrifuged and the pellet resuspension in 50 mM K2HPO4 buffer (pH 6.0) with containing 0.0005% hydrogen peroxide was as enzyme a substrate and 0.19 mg/mL of o-dianisidine chloride with a microplate reader at 460 nm. The results of MPO activity are expressed as OD 460nm/mg protein of lung tissue.

2.1.0. Western blot analysis

Lung tissue lysate was prepared by homogenization in RIPA lysis buffer with protease inhibitors, followed by centrifugation (12,000xg, 20 min) and protein were subjected to 10% SDS-PAGE. Electrophoresed proteins were transferred onto nitrocellulose membranes. After blocking, the membranes were incubated with primary antibody (1/2000 dilution). Appropriate HRP-conjugated secondary antibodies (Sigma, St Louis, MO, U.S.A.) were applied and the signals were detected by using the enhanced chemiluminescent method (Amersham International plc., Buckinghamshire, U.K.). The western blot was quantitated by analyzed using the Kodak Molecular Imaging Software (Eastman Kodak Company, Rochester, NY).

2.1.1. Statistical analysis

Values are expressed as the mean ± standard error of the mean. One-way analysis of variance (ANOVA) or Student's t-test was used to examine the differences among multiple groups or between two groups. ###p< 0.001 compared with the control group; *p < 0.05, **p < 0.01 and ***p < 0.001 significant compared to LPS alone group.

3. Results

3.1. GRh2 reduces LPS-induced histopathology changes of lung in mice

The morphology of the lungs was examined after LPS challenge. The results showed that the control group showed normal lung architecture (Fig. 1B). In the LPS-induced group of the lung specimens, neutrophils are infiltrating in the pulmonary vessel, edema of the interstitial space of the alveolar wall and those contexts causes alveolar epithelial cell damage. These improves of the pathological process by varying concentrations in GRh2 (5, 10 and 20 mg/mL) and Dex (10 mg/kg) groups mice, suggesting that GRh2 alleviated its pathological effects in LPS-induced ALI mouse model. Furthermore, the lung injury score showed GRh2 improved LPS induced inflammatory response (Fig. 1C). These results suggested that GRh2 evidenced by reduced inflammatory cell infiltration to protect the LPS challenge histopathological changes in the mice of lung tissues.

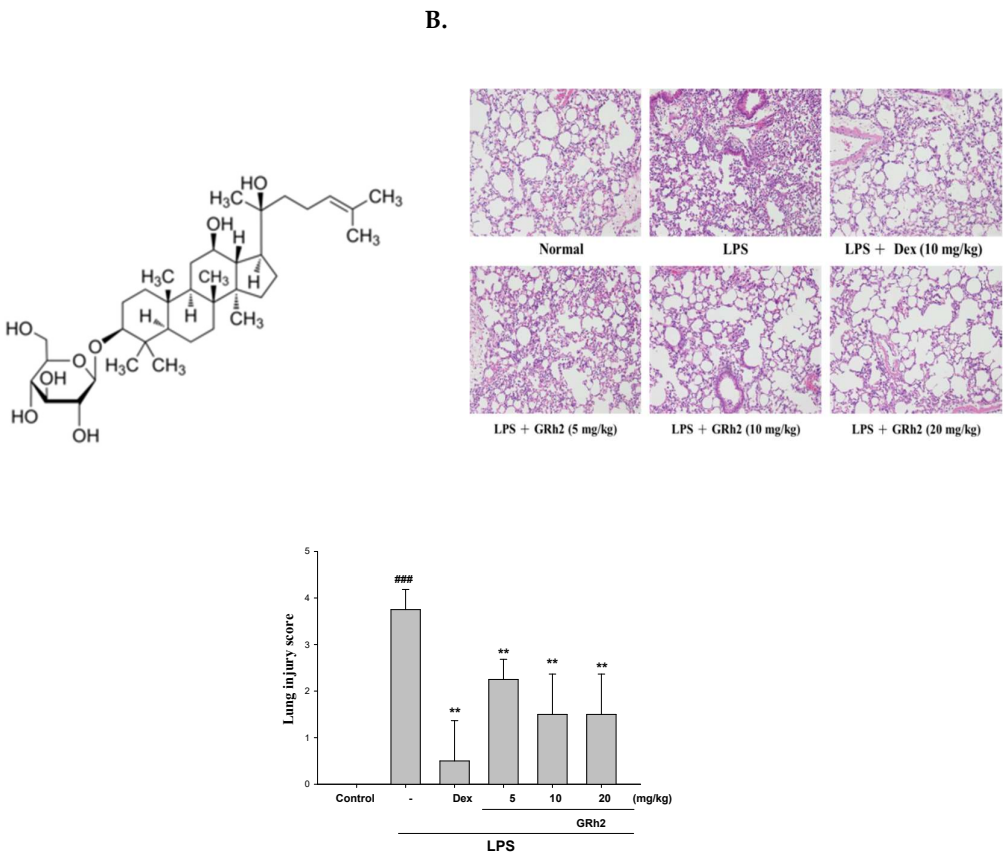

Figure 1. The chemical structure of ginsenoside Rh2 (GRh2) (A) and the effects of GRh2 on histopathological changes in the lung (B) and on the severity of lung injury were analyzed using the lung injury scoring system (C) in LPS-induced ALI mice. Mice were sacrificed 6 h after LPS stimulation. The left lungs were excised and embedded in 10% formalin, sectioned, and stained with H&E; magnification x400. Images are representative of three experiments. DEX: dexamethasone. The data are presented as the means ± S.E.M. ## compared with the control group. *$p < 0.05$ and **$p < 0.01$ compared with the LPS-alone group.

3.2. GRh2 decreases pulmonary wet/dry weight ratio and MPO activity by LPS challenge mice

Attenuating effect of GRh2 can be determined by pulmonary W/D ratio and MPO activity on LPS-induced lung injury. Under LPS stimulation improved the lung vascular permeability as a cause

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of edema compared to control group as indicated in the lung W/D ratio. However, GRh2 and Dex treatment could greatly reduce the lung W/D ratio (Fig. 2A) compared with the group treated with LPS alone. These results displayed that GRh2 could eliminate lung edema and pulmonary inflammation after LPS challenge.

Neutrophil infiltration can increase inflammatory responses and cell injury. However, MPO activity is a more useful index of neutrophil influx into lung tissue [15]. As Fig. 2B illustrates, mice were exposed by intratracheal instillation of LPS in lung tissues increased of MPO activities, significantly. When pretreatment with GRh2 (5, 10 and 20 mg/mL) and Dex, MPO activity was decreased in LPS treatment alone mice. These data revealed that GRh2 prevented pulmonary edema and lung tissue infiltration by LPS challenge mice.

3.3. GRh2 decreases total cells count and protein concentration by LPS challenge mice

LPS caused a markedly increase in total cells compared to the control group. However, pretreatment GRh2 or Dex decrease in the total cell count compared with treated LPS-induced ALI mice, significantly (Fig. 2C). Compared with data from a control group, total protein concentration in BALF decreased significantly after pretreatment GRh2 and Dex (Fig. 2D). These results recommended that inhibitory effect of GRh2 on ALI is connected with the attenuate leukocytes sequestration and the inflammatory response in the lung tissues.

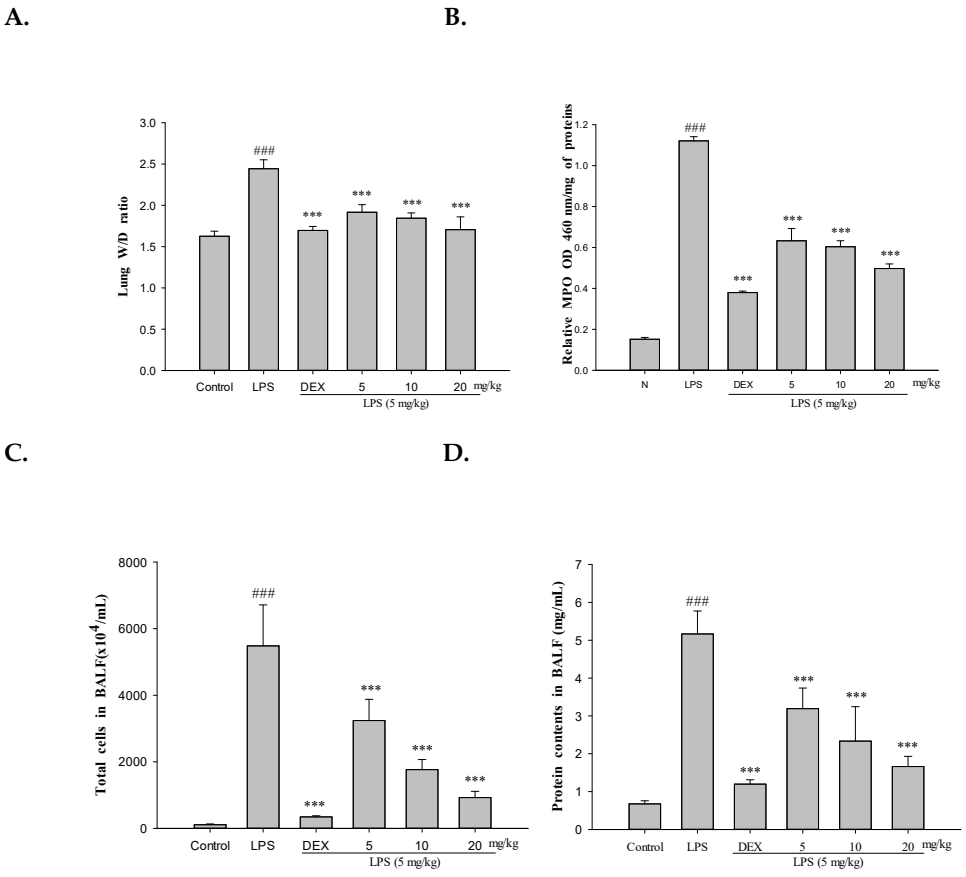

Figure 2. GRh2 improved pulmonary edema (W/D ratio) (A), Myeloperoxidase (MPO) activity (B) in *vivo* and reduced cellular counts (C) and total protein (D) in BALF. Six hours after LPS injection with or without GRh2 pretreatments, mice were sacrificed and their lungs were lavaged. The right lower lungs were used to assess wet to dry (W/D) ratio of lung. Cells in the BALF were collected and cytospin preparations were made. Total cells and total proteins in BALF were analyzed. Data represents mean ± S.E.M. of 6 mice. ###$p < 0.001$ were compared with sample of control group. * $p < 0.05$, ** $p < 0.01$ and*** $p < 0.001$, were compared with LPS-alone group.

3.4. GRh2 decreased the Level of Proinflammatory Cytokines by LPS challenge mice

Inflammatory mediator levels in tissues were detected by ELISA assays. LPS stimulated ALI mice significantly increased the NO, TNF-α, IL-1β, IL-4, and IL-6 levels in the BALF compared to control group, significantly (Fig. 3A, 3B, 3C, 3D and 3E). GRh2 and Dex treatment ameliorated the NO, TNF-α, IL-1β, IL-4, and IL-6 production after LPS challenge. Furthermore, GRh2 treated group in LPS-induced ALI BALF increases IL-10 concentration, significantly (Fig. 3F).

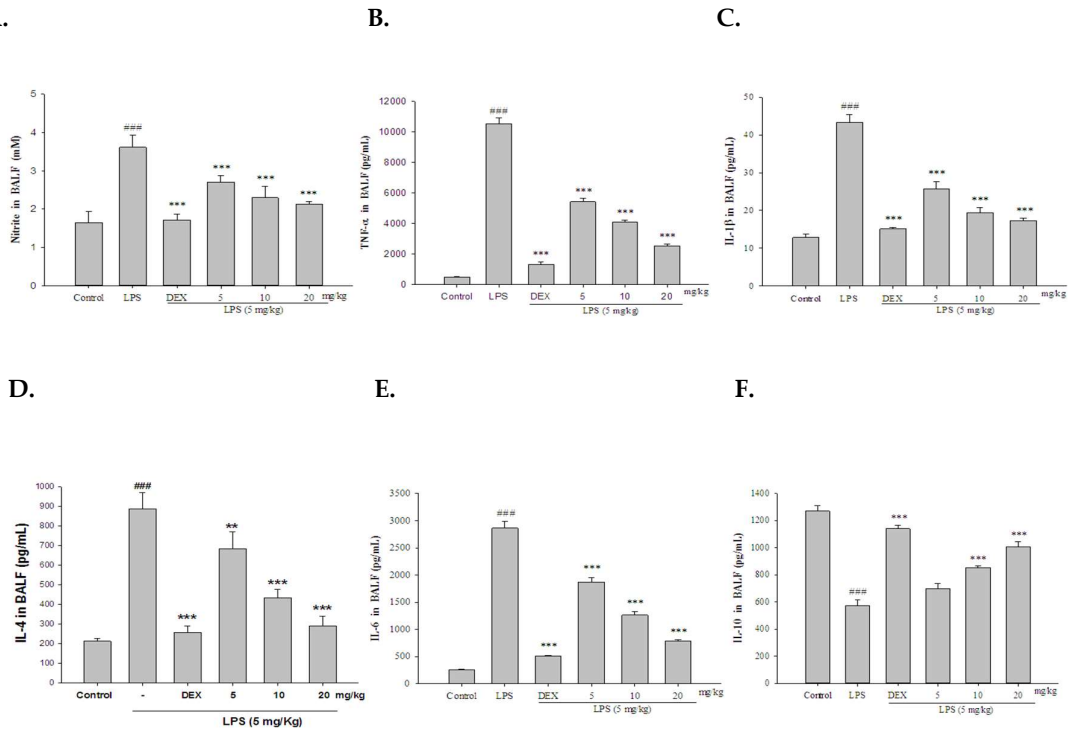

Figure 3. GRh2 down regulated NO (A), TNF-α (B), IL-1β(C), IL-4 (D), IL-6 (E), and increased IL-10 (F) in BALF. Six hours after LPS injection with or without SS pre-treatments, mice were sacrificed, their lungs were lavaged and the BALF were collected. NO, TNF-α, IL-1β, IL-4, IL-6 and IL-10 were detected by ELISA. Data represents mean ±S.E.M. of 6 mice. ###p < 0.001 were compared with sample of control group. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$, were compared with LPS-alone group.

3.5. GRh2 Inhibited LPS-induced ALI iNOS and COX-2 and inactivating NF-κB and IκBα protein expressions

To determine whether pretreatment with GRh2 inhibited NO production was regulated by iNOS and COX-2 protein expressions after LPS challenge. The results revealed that the pretreatment GRh2 inhibits the protein expression of iNOS and COX-2 after LPS challenge of lung tissues (Fig. 4A).

The signal pathways cause the NF-κB accumulation in the nuclear, which can be activated by a variety of stimuli with pro-inflammatory cytokines: TNFα and IL-1β [6]. Our study demonstrates that GRh2 treatment inhibited the IκBα and NF-κB degradation by LPS challenge in mice (Fig. 4B). Thus, GRh2 attenuates by regulating the NF-κB signaling pathway after LPS challenge.

3.6. GRh2 suppressed the MAPK pathway activation by LPS challenge mice

As shown in Fig. 4C, we found that the phosphorylation of MAPKs protein was activated after LPS challenge in mice. More, the expression of phosphorylated ERK, JNK and p38 decreased when pretreatment GRh2 and Dex. These results revealed that GRh2 suppressed the expression of MAPK proteins after LPS challenge.

A. **B.** **C.**

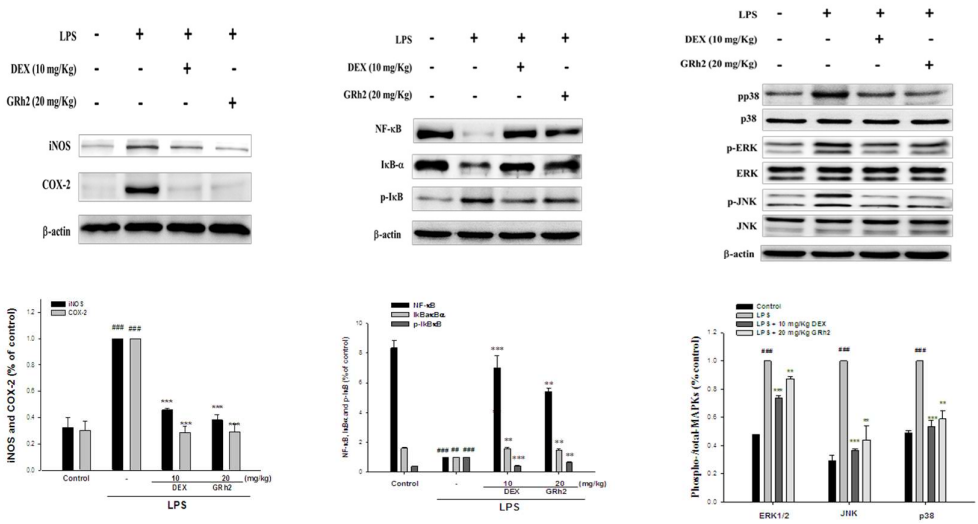

Figure 4. Effects of GRh2 on LPS-induced iNOs, COX-2 (A), IκB-α, NF-κB (B) and MAPK phosphorylation (C) protein expression in ALI mice of lung tissues. Mice were pretreated with different concentrations of GRh2 for 1 h and stimulated with LPS. The Western blotting by using an antibody specific were used for the detection of iNOs, COX-2, IκB-α phosphorylated, NF-κB nuclear and cytosol, and total forms of three MAPK molecules, ERK, p38, and JNK. Data represents mean ± S.E.M. of 6 mice. ## $p< 0.01$ and ### $p< 0.001$ were compared with sample of control group. ** $p< 0.01$ and*** $p < 0.001$ were compared with LPS-alone group.

3.7. GRh2 Attenuates LPS-induced oxidative stress and HO-1/Trx-1/KAP-1/Nrf2 signal pathway by LPS challenge mice

Oxidative stress elevated the levels of reactive oxygen species that cause the tissue injury. SOD is a class of related enzymes that catalyzed the breakdown of superoxide anion, and the activity of SOD also has reduced in LPS-induced ALI mice [7]. The LPS administration alone reduced the activity of catalase, SOD and GPx and the anti-oxidative relative protein expressions such as HO-1, Trx-1, KAP-1 and Nrf2 and elevated Keap1 protein expression when compared to a control group (Fig. 5A). However, GRh2 elevated the antioxidant enzymes activities and the anti-oxidative relative protein expressions compared to the LPS alone group. These results demonstrated that GRh2 improved the expression of anti-oxidative enzymes related proteins after LPS challenge.

3.8. GRh2 attenuated the TLR4/PI3K/Akt/mTOR signaling by LPS challenge mice

As shown of Fig. 5B, LPS alone group lead to increased expression of TLR4, PI3K, Akt and mTOR protein expressions compared with control groups. Otherwise, GRh2 treatent group inhibit the expression of TLR4/PI3K/Akt/mTOR signal transduction pathway compared to LPS-induced group. These results denoted that the protective effect of GRh2 after LPS challenge through inhibite the proteins expression of TLR4/PI3K/Akt/mTOR signal pathway.

3.9. GRh2 Activated the RAF/MEK pathway by LPS challenge in mice

Growth factors use the Raf/MEK signaling cascade to transmit signals to regulate protein expression. In this study, LPS alone group increase the phosphorylation of RAF and MEK protein expression when compared to the control group (Fig. 5C). On the contrary, GRh2 administration treated group exhibited significantly inhibited activity of the RAF/MEK pathway when compared with the group treated with LPS alone. These results recommended that GRh2 inhibited the RAF/MEK pathways after LPS challenge.

A. **B.** **C.**

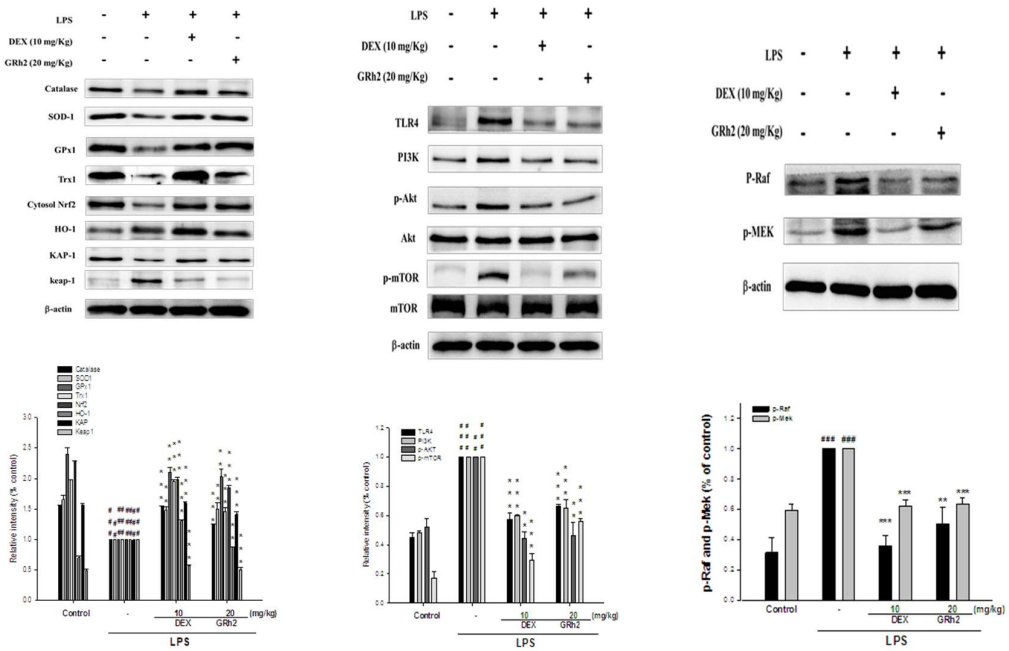

Figure 5. Effects of GRh2 on LPS-induced anti-oxidative enzymes (catalase, SOD and GPx)、HO-1, Trx-1, Nrf2/Keap1, KAP1(A), TLR4, PI3K, Akt, mTOR (B), p-Raf-1 and p-Mek (C) protein expression protein expression in ALI mice of lung. Mice were pretreated with different concentrations of GRh2 for 1 h and stimulated with LPS. The Western blotting by using an antibody specific were used for the detection of catalase, SOD, GPx, HO-1, Trx-1, Nrf2/ Keap1 and KAP1 protein expression. Data represents mean ±S.E.M. of 6 mice. ##p< 0.05 and ### p< 0.001 were compared with sample of control group. ** p< 0.01, and*** p < 0.001, were compared with LPS-alone group.

3.1.0. Blocking RAF Synergy with GW-5074 to Increase Anti-inflammatory Capacity of GRh2 by LPS challenge mice

To determine whether Raf-1 kinase inhibitor (GW-5074) could suppress Raf-1/MEK/ERK pathways or not, we investigated the patterns of related protein expression of Raf-1/MEK/ERK pathway by LPS challenge mice treatment with GW-5074 (2.0 mg/kg). The effects of LPS-induction pro-inflammatory cytokines release (NO, TNF-α, IL-1β and IL-6) were suppressed by GW-5074 inhibitor. In addition, inhibition of the induction of pro-inflammatory cytokines by co-treatment of GRh2 and GW-5074 compared to the LPS alone group (Fig. 6A-D). Furthermore, co-treatment of GRh2 with GW-5074 increased the level of IL-10 concentration in BALF, significantly (Fig. 6E). These results indicated that GRh2 suppressed the activity of Raf-1/MEK/ERK pathways by LPS challenge ALI mice.

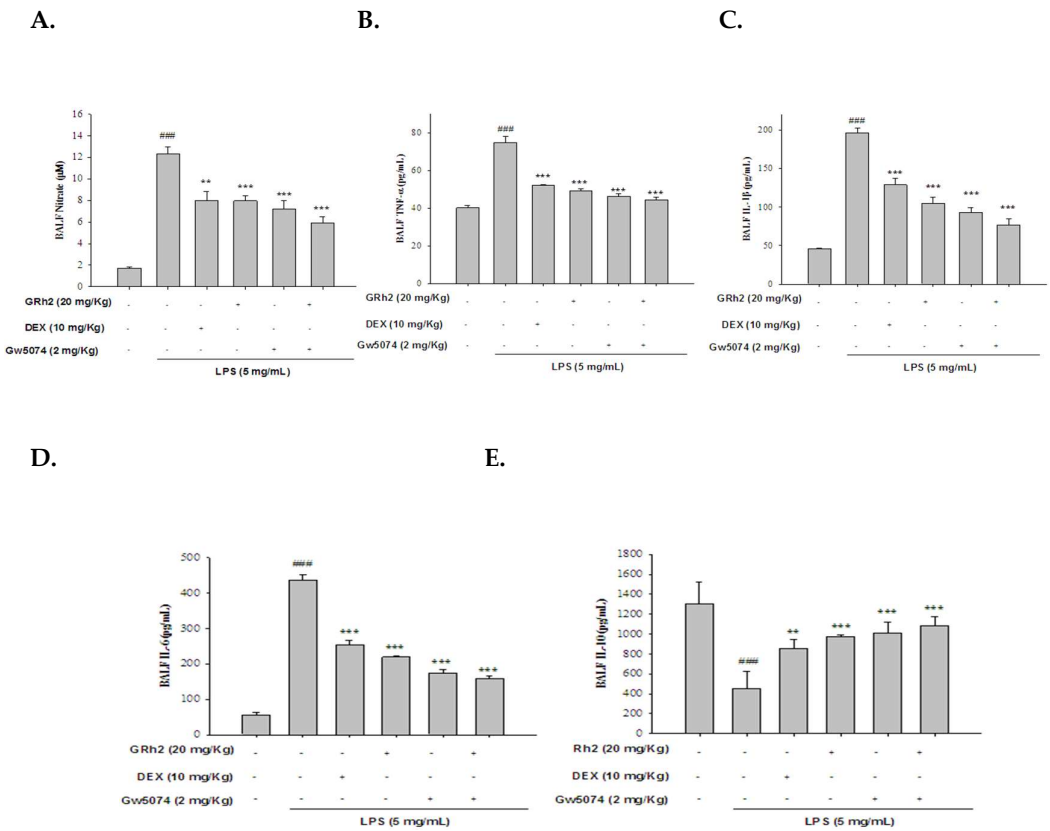

Figure 6. GRh2 and Raf-1 inhibitor (GW-5074) reduced cellular counts (A), total protein (B), TNF-α (C), IL-6 (D), IL-1β (E), NO (F) in BALF and down regulated iNOS and COX-2 protein expression (G) in lung. Mice were pretreated with different concentrations of GRh2 and TAK-242 for 1 h and stimulated with LPS. The Western blotting by using an antibody specific were used for the detection of iNOS, COX-2, NF-κB nuclear and cytosol. Six hours after LPS injection with or without GRh2 and GW-5074 pre-treatments, mice were sacrificed, their lungs were lavaged and the BALF were collected. TNF-α, IL-6, IL-1β, NO and IL-10 were detected by ELISA. Data represents mean ±S.E.M. of 6 mice. ### p< 0.001 compared with sample of control group. *** p< 0.001, were compared with LPS-alone group.

4. Discussion

In this study, we induce an animal model of ALI using intratracheal instillation of LPS challenge to confirm the protection effect of anti-inflammatory of GRh2. LPS elicited causes significant and dose-related increases the pro-inflammatory cytokines production, which occurs after 2-6 h to maximize 24 h at least [16]. Currently, glucocorticoids are a class of steroid hormones that may be treatable with anti-inflammatory medicines that would be used in the treatment of most patients with ALI. Therefore, the positive control of Dex estimates the efficacy of anti-inflammatory agents GRh2 by LPS challenge ALI mice. Nonsteroidal anti-inflammatory drug is one of the most commonly used drug classes that reduce pain and inflammation. Moreover, the discovery and development of anti-inflammatory drugs must base on their effects on signal transduction and as anti-cytokine agents [17, 18]. Thus, we found that treatment GRh2 reduced the LPS challenge lung edema, neutrophil infiltration, the secretion of proinflammatory cytokines in the BALF and regulation of the Raf-1/MEK/ERK and Keap1/Nrf2/HO-1 signaling pathways. This study indicates that GRh2 exhibits anti-inflammatory and antioxidant activity after LPS challenge.

It is characterized of ALI by a disruption of the integrity and function of the endothelial and epithelial barriers, pulmonary edema, release of pro-inflammatory cytokines and inflammatory mediators and large numbers of neutrophils infiltration [19]. LPS challenge ALI mice model have

been extensively used to study the pathological processes and states in the scientific research [20]. Thus, the animal model of ALI induced by administration of LPS through the trachea is suitable for the study of potential human primary prevention or treatment drugs.

LPS-induced ALI induces a large number of pro-inflammatory mediators to recruit neutrophils to the lungs [21]. In the early stages of inflammatory response, neutrophils are the major class of immune cells that destroy the alveolar capillaries. Ultimately, neutrophils infiltration dysfunction lead to hypoxia and pulmonary edema associated with the formation of hyaline membrane in alveolar walls [22]. The clinical pathological features of ALI in humans are similar to LPS challenge ALI in murine model [23]. However, the search for an effective pharmacologic therapy for ALI is still ongoing. Therefore, we used intratracheal instillation to study the role of GRh2 in the LPS challenge ALI mouse model in order to establish a new drug for ALI. Currently, we found that GRh2 alleviated the LPS-induced histopathological changes (infiltration of proinflammatory cells and lung oedema).

LPS-induced ALI is a main pathogenesis of ALI by the infiltration of leukocyte and macrophage migration into lung tissues [24]. In the current study, GRh2 treatment markedly reduced the total cell numbers compared to LPS treated animals. In addition, MPO is the most abundant granule enzyme in the neutrophil and plays a central role involved with inflammatory disorders. The measurement of MPO activity is a quantitative assessment for evaluating neutrophils infiltration into lung parenchyma or alveolar spaces [25].

Cytokine are a novel superfamily of related low-molecular-weight proteins that mediate many of the immune reactions where the cytokine signals are amplified [26]. Pro-inflammatory cytokines are released by activated macrophages play a critical early role in inflammatory diseases and demonstrate in several clinical studies. Therefore, ALI is caused by many of pro-inflammatory cytokines including TNF-α, IL-1β, IL-4, IL-6 and IL-10 and other anti-inflammatory cytokines mediators [26]. Increased levels of pro-inflammatory cytokines have been observed in ALI patients and are associated with major inflammatory disorders [27]. TNF-α is a key cytokine exerting pleiotropic effects and can induce a pro-inflammatory response by high particle concentrations. In addition, The IL1 and its related family member are mainly proinflammatory cytokines associated with the pathogenesis of acute and chronic disorders [28]. In addition, IL-1β stimulates the production of other cytokines in the inflammatory response and modulates the proinflammatory cytokines cascade in ALI [29]. IL-6 is released by monocytes and macrophages in response to other cytokines in the inflammatory response disease. In the clinical test, the higher levels of IL-6 are increased in ALI patients of serum that predict increased mortality [30]. And after LPS instillation increase IL-4 content in BALF at 24 h and IL-4 expression are correlated with the deposition of extracellular matrix and lung fibrosis [31]. These results presented that remodeling processes often cause unregulated fibro-proliferation and fibrosis in the late phase of ALI [25]. In addition, the major function of IL-10 strongly inhibits the production of pro-inflammatory cytokines. Recent studies have suggested that IL-10 protects against lethality during endotoxemia-induced shock in mice [32]. The IL-10 promoters differ in the absence of an NF-κB binding site and the presence of a cyclic AMP response element [32]. In our study, the levels of TNF-α, IL-1β, IL-4 and IL-6 were dramatically attenuated and elevate IL-10 levels by GRh2 in BALF.

The cells contain a number of antioxidant enzymes (SOD, catalase and GPx) to prevent cells or tissues damage that expression of antioxidant enzymes attempt to diminish the oxidative stress. SOD is an extremely efficient enzyme that catalyzes the partitioning of the superoxide anion to hydrogen peroxide and oxygen, while catalase and GPx catalyze the hydrogen peroxide to form oxygen and water [10]. In this study, we showed that GRh2 increases the antioxidant protein activity (SOD, catalase and GPx) in the ALI model. In addition, studies have shown that Keap1 is a key sensor for oxidative stress. Under oxidative stress, Nrf2 is released from Keap1 that activated Nrf2 and its downstream regulated genes in the nucleus and correlated with induction of HO-1, GPx, glutathione-S-transferase and Trx-1, allow to scavenging the free radicals in cells from oxidative damage [15]. The function of Nrf2 is maintaining cellular homeostasis through its ability to regulate the antioxidant proteins, detoxification enzymes and other stress response proteins [15]. In addition, PI3K and MAPK signaling are also connected in the activation of Nrf2 [33]. The results indicated that

protective effects of GRh2 regulated the Keap1/Nrf2/HO-1 signal pathway against oxidative stress. Nrf2 is a key molecule involved in targeting specific proteins NF-κB pathway, which may be associated with the inflammatory regulation.

NF-κB is a family of transcription factors which serves as a signal regulator in inflammation, cell proliferation, and differentiation [15]. Activation of NF-κB was reported to increase with ALI in clinical test and NF-κB p65 overexpression of ALI patients in the alveolar macrophages caused by severe infection compared to the control group [7]. In addition, MAPK cascades have proved to play a vital role in transduction extracellular signals to cellular responses such as inflammatory cytokine production induced by LPS.

The MAPK activation immediately after LPS, the levels of the phosphorylated of MAPKs (ERK1/2, p38 and JNK) was increased [12]. In this study, GRh2 suppressed the phosphorylation of MAPKs in LPS challenge ALI mice. GRh2 also suppressed the TNF-α, IL-1β, and IL-6 productions through NF-κB activation because they can control pro-inflammatory cytokine expressions in LPS-induced model. In this research, we discovered that GRh2 significantly inhibited IκB-α degradation and the phosphorylation of NF-κB and MAPK by LPS challenge mice.

The PI3K/AKT/mTOR signal plays an active part in the regulating several of cellular functions, including cell proliferation and apoptosis and is critically modulated TLR signaling pathways [33]. This result showed that inflammatory response of LPS-induced ALI was mediated through TLR4 receptor which increased the level of PI3K, p-Akt and p-mTOR protein expressions. The results of our studies confirm that the PI3K/AKT/mTOR pathway is a potential predictive marker for GRh2 treatment by LPS challenge mice.

The activation of the ERK pathway is caused by various upstream stimuli that accumulate on the Ras family of small G proteins [16]. Activated Ras then engages with its downstream effectors of a series of kinases from RAF to MEK to MAPK is an example of a protein kinase cascade. Raf-1 is a serine threonine kinase that phosphorylates and activates a family of protein kinases termed MAP kinase or Mek [34]. It is thought that the Ras/Raf-1/MAP pathway play an active role in modulation of cell survival, proliferation and differentiation.

In this study, our results clearly show that TLR4 mediate LPS-induced activation of ERK1/2 proteins via the Ras/Raf-1/MAP kinase. In addition, the activation of Raf-1 is quick and leads to the activation of Mek. Moreover, based on our studies in the Raf-1 kinase inhibitor, LPS participates in TLR4 induction of the canonical Raf/Mek/ERK pathway. Multiple points of exists between the PI3K/Akt/mTOR and Raf/Mek/ERK signaling. For example, the activation of NF-κB is associated with the TNFα receptor and the Ras-Mek-ERK pathways are coupled to regulate NF-κB dependent production of pro-inflammatory mediators [34, 35]. Therefore, these results demonstrated that Raf/Mek/ERK signaling pathways were stimulated in this LPS challenge ALI animal model, and blocking this pathway with GRh2 treatment.

5. Conclusions

In conclusion, our study showed that GRh2 regulate inflammatory responses in LPS challenge animal model via inhibiting lung pathologic changes, lung edema, inflammatory cell infiltration, the release of a variety of pro-inflammatory cytokines (TNF-α, IL-1β, IL-4 and IL-6) and elevated the release of IL-10. Together, research data suggested that GRh2 has potent anti-inflammatory response by inhibited the TLR4/ PI3K/Akt/mTOR, Raf-1/MEK/ERK and Keap1/Nrf2/HO-1 signaling pathways (Fig. 7). Therefore, GRh2 exerts anti-inflammatory effects in *vivo* and still requires more comprehensive research before realizing the full clinical application of the drug.

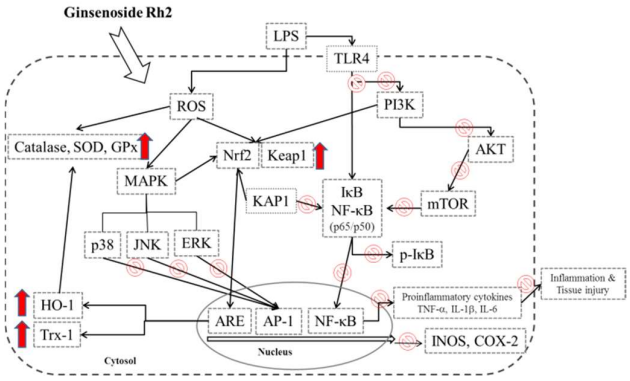

Figure 7. The schemes of the mechanism for the protective effect of GRh2 on LPS-induced inflammation.

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