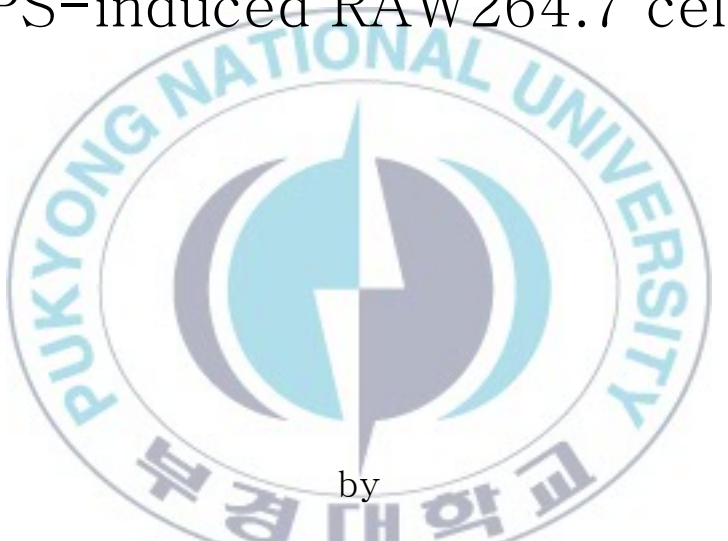
Thesis for the Degree of Master of Science

Toluhydroquinone inhibits inflammatory mediators through NF-κB and MAPKs pathways in LPS-induced RAW264.7 cells



by

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August 2013

Toluhydroquinone inhibits inflammatory mediators through NF-κB and MAPKs pathways in LPS-induced RAW264.7 cells

(LPS 로 활성화된 RAW264.7 세포에서의 NF-κB 와 MAPK 신호 전달을 통한 toluhydroquinone 의 면역 매개 인자 저해 효과)

Advisor: Prof. Gun Do Kim

by
Duk Hyun Hwang

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Toluhydroquinone inhibits inflammatory mediators through NF-κB and MAPKs pathways in LPS-induced RAW264.7 cells

A dissertation

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CONTENTS

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Abstract

Chemotherapeutic agents from several metabolites are used to treat many diseases. There are diverse marine organisms such as invertebrates, algae, bacteria, and fungi, which produce secondary metabolites. Toluhydroquinone is a secondary metabolites product of marine fungi *Phoma herbarum* and its bioactivity is rarely known. The purpose of this study is to investigate the anti-inflammatory mechanism of toluhydroquinone in lipopolysaccharide (LPS) -induced RAW264.7 cells. Toluhydroquinone inhibited nitric oxide (NO) production and prostaglandin E_2 (PGE_2), as well as reduced inflammatory mediator proteins including inducible nitric oxide synthase (iNOS) and cyclooxyganse-2 (COX-2) expression. Toluhydroquinone decreased the pro-inflammatory cytokines expression of tumor necrosis factor-α (TNF-α), interleukine-1β (IL-1β), and interleukin-6 (IL-6). Additionally, toluhydroquinone inhibited the phosphorylation of nuclear factor-κB (NF-κB).

Also, toluhydroquinone reduced the phosphorylation of mitogen-activated protein kinases (MAPKs) pathway, which is involved extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38. Moreover, toluhydroquinone induced heme oxygenase-1 (HO-1) protein expression which is stress-inducible enzyme to effect cell protection, through nuclear factor E2-related factor 2 (Nrf2). These results indicate that the mechanism of toluhydroquinone effects on anti-inflammatory in LPS-stimulated RAW264.7 cells.

INTRODUCTION

Inflammation is an important defense system against injury or microbial pathogens to protect body. Inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) react to fix and regenerate the tissue (Chen *et al.,* 2013; Oh *et al.,* 2012).

The PGE_2, one of the inflammatory mediators, is produced while the cyclooxygenase converts to arachidonic acid (Lee *et al*., 2012). The pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 regulate immune system, induce inflammatory diseases and control regenerate system. However, when the inflammatory mediators are over-expressed, those induce many immune diseases and cause cancer, circular diseases, or autoimmune disorders (Kim *et al*., 2012b; Part *et al*., 2013a; Tang *et al*., 2011). Therefore, inflammatory mediators play an important role in the pathogenesis of many inflammatory diseases (Yoo *et al*., 2012).

Macrophages function as an important part in inflammatory response. They are primary pro-inflammatory cells and offers first defense system to protect the body from the stimuli such as lipopolysaccharide (LPS). LPS acts

endotoxin in the body and it is from the cell wall of gram negative bacteria (Rehman *et al*., 2012; Tuntipopipat *et al*., 2011). It promotes inflammatory cytokines in diverse cells and causes acute inflammatory response (Ngkelo *et al*., 2012). In macrophages, there are several pathways involving LPS-related signaling. Nuclear factor kappa B (NF-κB), mitogen activated protein kinases (MAPKs), and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathways are representative pathways (Kim *et al*., 2012c).

During the oxidation of L-arginine to L-citrulline, NO production is occurred and regulated by NOS (Kim *et al*., 2012a). NO synthase (NOS) has three isoforms including inducible NOS (iNOS), epithelial NOS (eNOS), and neuronal NOS (nNOS) (Hu *et al*., 2009). iNOS is not produced in normal mammals, but it is synthesized by cytokines or LPS through macrophage (Borutaite *et al*., 2006). NO production is related several inflammatory diseases (Lee *et al*., 2007). Thus, reduction of NO production is shown one way of curing inflammatory-related diseases (Kwon *et al*., 2012).

MAPKs involve in extracellular signal regulated kinase (ERK), c-jun N terminal kinase (JNK), and p38. These pathways respond extracellular stimuli and control cellular responses such as the production of inflammatory cytokine mitosis, differentiation, and cell survival/apoptosis.

Phosphorylation of MAPKs leads in response to activation of transcription factors (Park *et al*., 2013b). Accordingly, NF-κB and MAPKs pathways are regulated to immune response.

Inflammatory mediators activate NF-κB pathway (Jin *et al*., 2012). Without stimulation, NF-κB is inactivated by combination with inhibitory kappa B (IκB) family (Wullaert *et al*., 2011). However, upon stimulation, the IκB kinase (IKK) complex phosphorylates IκBα, the consequence is that IκBα is degraded and NF-κB is translocated into the nucleus (Yang *et al*., 2013). It allows to bind the promoter area and to offer the transcription of inflammatory-related gene (Chow *et al*., 2012).

Heme oxygenase-1 (HO-1) is considered as a stress-inducible enzyme and degrades heme to biliverdin, carbone monoxide (CO), and free iron. Biliverdin reductase catalyzes biliverdin to bilirubin (Zeng *et al*., 2010). HO-1 protects cells from stress environment and regulates inflammatory responses against pro-inflammatory stimuli (Abuarqoub *et al*., 2006). HO-1 is induced through MAPKs and PI3K/Akt in response to anti-inflammatory responses. Also, HO-1 is mediated NF-E2 related factor-2 (Nrf2) (Kim *et al*., 2012d), which is a transcription factor; controls antioxidative stress (Wang *et al*., 2010).

The development of marine environment has lasted to find products that have unique structure and biologically active natural products, which has known as secondary metabolites (Leutou *et al*., 2012). There are diverse marine organisms such as invertebrates, bacteria, algae, and fungi and those offer secondary metabolites. Many diseases are treated chemotherapeutic agents from several secondary metabolites (Whibley *et al*., 2007).

Endophytic fungus interact with their host and provide bioactive metabolites (Zhang *et al*., 2011). A diverse endophytic fungi produces secondary metabolites which effect anti-microbial, anti-cancer, and other bioactivities (Cui *et al*., 2012). *Phoma herbarum* is one of the endophytic fungus and it is isolated from *Hypnea saidana* (Suetrong *et al*., 2009). *Phoma herbarum* Westend. (Fungi imperfecti) produces diverse secondary metabolites such as diterpenoid plant hormone gibberellins (Hamayun *et al*., 2009), naphthalenone, nitrophthalic acid, nonenoide, terpenoid, and polyketide (Zhang *et al*., 2013). Toluhydroquinone is one of the secondary metabolites, from *Phoma herbarum*, and it affects anti-oxidant (Suetrong *et al*., 2009).

In the present study, the anti-inflammatory mechanism of toluhydroquinone in LPS-induced RAW264.7 cells was investigated by measuring NO assay and examining the expression of pro-inflammatory cytokines. Also, we

evaluated whether toluhydroquinone effects phosphorylation of NF-κB and MAPKs pathways and induction of HO-1 protein. Our results suggest that toluhydroquinone inhibits inflammatory mediators such as NO, PGE_2, TNF-α, IL-1β, and IL-6 through NF-κB and MAPKs pathways in LPS-stimulated RAW264.7 cells.

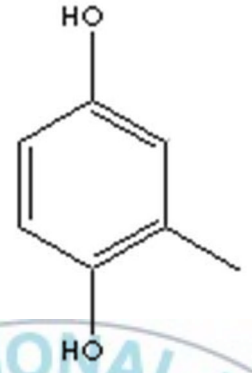

Fig. 1 Chemical structure of toluhydroquinone, isolated from *Phoma herbarum*.

MATERIALS AND METHODS

Fungal isolation and culture

The fungal strain, *Phoma herbarum* was isolated from the surface of the marine red algae *Hypnea saidana* collected in the Tongnyeong, Gyeongnam province, Korea in 2008 and identified based on the morphological evaluation. A voucher specimen is deposited at Pukyong National University with the code MFA301-1.

The fungus was cultured (20 L) for 21 days (static) at 29℃ in SWS medium: soytone (0.1%), soluble starch (1.0%), and seawater (100%).

Extraction and isolation

The culture broth and mycelium were separated, and the broth (10 L) was extracted with ethyl acetate to provide a crude extract (640 mg) which was subjected to silica gel flash chromatography and progressively eluted with n-hexane/EtOAc (5:1), n-hexane/EtOAc (1:1), n-hexane/EtOAc (1:5), n-hexane/EtOAc (1:10), and finally with EtOAc. Each collection (30 mL each) was combined on the basis of their TLC profiles to yield five major fractions.

Medium pressure liquid chromatography (MPLC) of each fractions on ODS by elution with MeOH afforded compounds 1-5, respectively. The isolated compounds were further purified by HPLC (YMC ODS-A, MeOH) utilizing a 30 min gradient program of 50% to 100% MeOH in H_2O to furnish (+)-epoxydon (1, 5.0 mg), (+)-epoxydon monoacetate (2, 12.0 mg), gentisyl alcohol (3, 10.0 mg), 3-chlorogentisyl alcohol (4, 20.0 mg), and methylhydroquinone (toluhydroquinone) (5, 5.0 mg), respectively. The isolated fraction such as methylhydroquinone (toluhydroquinone) was used in this study.

Physicochemical data of compound

Common name: Toluhydroquinone (methylhydroquinone). $CH_3C_6H_3(OH)_2$ (M.W. 124.14) (Fig. 1). ^{1}H NMR (400 MHz, DMSO-d_6) 8.44 (1H, s, 2-OH), 6.53 (1H, d, J = 8.5 Hz, H-3), 6.36 (1H, dd, J = 8.5, 2.8 Hz, H-4), 8.48 (1H, s, 5-OH), 6.45 (1H, d, J = 2.8 Hz, H-6), 2.02 (3H, s, H_3-7); ^{13}C NMR (100 MHz, DMSO-d_6) 124.3 (s, C-1), 147.6 (s, C-2), 117.1 (d, C-3), 115.0 (d, C-4), 149.5 (s, C-5), 112.6 (d, C-6), 16.1 (q, C-7).

Drugs and reagents

Stock concentration of Toluhydroquinone (50 mM) was prepared with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20℃. The stock solution was further diluted with the appropriate assay medium immediately before use. The maximum final concentration of DMSO (< 0.1%) did not affect cell proliferation.

Cell culture

RAW264.7 mouse macrophage cells and human embryonic kidney HEK293 cells were obtained from the American Tissue Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose with L-glutamine (Cellgro Mediatech, Inc., Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cellgro), and 1% penicillin-streptomycin (PPA Laboratories GmbH, Pasching, Austria). The cells were incubated in humidified atmosphere with 5% CO_2 at 37℃.

Cell viability assay

To measure the cell viability, RAW264.7 cells and HEK293 were seeded in

96-well plates at 1 x 10^5 cells/well. After 24 h incubation, cells were treated with various concentration of toluhydroquinone (0 – 10 uM) for 2 h, followed by stimulation with LPS (1 ug/ml) for 24 h. The medium was removed and 100 ul DMEM added to each well. 10 ul of WST-1 (Daeil Lab service, Seoul, Korea) was added and incubated for additional 3 h. The absorbance at 460 nm was measured with ELISA reader (Molecular Devices, Silicon Valley, CA, USA). The inhibitory rates were calculated.

Nitric Oxide (NO) assay

RAW264.7 cells (5 x 10^5 cells/well) were cultured in 24-well plates for 24 h. The cells were pretreated with Toluhydroquinone (0 – 10 uM) for 2 h, and then treated with LPS (1 ug/ml) in the presence or absence of Toluhydroquinone for 24 h. NO in the culture medium was examined as an indicator of NO production by Griess reagent (Sigma-Aldrich, St. Louis, MO, USA). The Griess reagent was mixed with the same volume of 100 ul culture medium and incubated at room temperature for 10 min. The absorbance at 540 nm was examined with ELISA reader.

Enzyme-linked immunosorbent assay (ELISA)

The cells were treated with Toluhydroquinone for 2 h, and induced with LPS (1 ug/ml) for 24 h. After incubation, the culture medium was harvested and the level of PGE_2, IL-1β, IL-6, and TNF-α was analyzed using ELISA assay kit (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. The absorbance was measured at 450 nm using ELISA reader.

Western blot analysis

RAW264.7 cells were treated, washed with ice-cold 1 x phosphate-buffered saline (PBS) and harvested by cell scraper. And then, the harvested cells were collected by centrifugation, lysed with ice-cold lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS and cocktail of proteinase inhibitors (PMSF, EDTA, aprotinin, leupeptin, and prostatin A) (Intron biotechnology, Gyeonggi, Korea). After incubation for 30 min in ice, cell debris was removed by centrifugation at 14,000 rpm for 20 min at 4℃. The protein concentration was examined using a Protein Quantification Kit (CBB solution®) (Dojindo Molecular Technologies, Rockvile, MD, USA) with

bovine serum albumin (BSA) as standard. An aliquot from each sample was boiled with sample buffer for 5 min, and then resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrotransferred to a nitrocellulose membrane (PALL Life Sciences, Pensacola, MI, USA) and blocked with 5% skim milk in PBST buffer (4.3 mM $NaPO_4$, 1.4 mM KH_2PO_4, 135 uM NaCl, 2.7 mM KCl, and 0.5% Tween-20). After blocking, the membrane was probed with primary antibodies ((anti-iNOS, anti-COX-2, anti-TNF-α, anti-p-Akt, anti-Akt, anti-p-p38, anti-p38, anti-p-ERK1/2, anti-p-JNK, anti-JNK, anti-Ras, anti-p-c-Raf, anti-p-MEK1/2, anti-TRAF2, anti-RIP, anti-p-MKK3/MKK6, anti-p-MAPKAPK-2, anti-p-SEK1/MKK4, anti-NF-κB, anti-p-IκBα, anti-IκBα, anti-IKKα, anti-HO-1, anti-GAPDH, and anti-β actin; Cell Signaling Technology Inc., Beverly, MA, USA), (anti-IL-1β, anti-IL-6, anti-ERK1/2, and anti-p-NF-κB; Santa Cruz, CA, USA), and anti-Nrf2; abcam, Cambridge, UK) and washed three times with PBST buffer. The membrane was followed by incubation for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG or anti-rat IgG as second antibodies (Cell Signaling Technology Inc.). The blots were then washed with PBST buffer and were visualized by an enhanced chemiluiminescent (ECL) detection solution

(AbFontier, Gyeonggi, Korea).

Immunofluorescent staining

Cells grown on coverglass-bottom dishes (SPL Lifesciences, Gyeonggi, Korea) were incubated and treated with 1uM of the Toluhydroquinone for 2 h and induced LPS for 24 h. For this, cells were pretreated with DAPI solution for 30 min at 37℃ and then fixed with 4% formaldehyde (Sigma, St. Louis, MO, USA) at room temperature for 15 min. Samples were followed by blocking for 1 h in 5% rabbit normal serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) of host primary antibodies with 0.3% Triton X-100. Fixed and blocked cells were incubated with 0.1 ug/ml of primary antibody (NF-κB p65) for 2 h, and then with 0.1 ug/ml of anti-rabbit IgG (H+L), F (ab') 2 Fragment (Alexa Fluor® 488 Conjugate) (Cell Signaling Technology Inc.) for another 1 h. The stained cells were mounted on the slides with Prolong Antifade Reagent (Invitrogen, Eugene, OR, USA) and observed in a fluorescent Nikon ECLIPS 50i microscope equipped with charged-coupled device (CDD) camera (Nikon, Tokyo, Japan). Images were captured and processed with a High-Content Analysis Software (Cambridge Healthtech Institute, Needham, MA, USA).

Statistical analysis

The GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA) for window was used to analyze the statistic significance of differences. Determinations were performed in triplicates and the results are expressed as mean ± SD. ANOVA post hoc test and subsequently Dunnett's multiple comparison tests were used for statistic analysis.

RESULTS

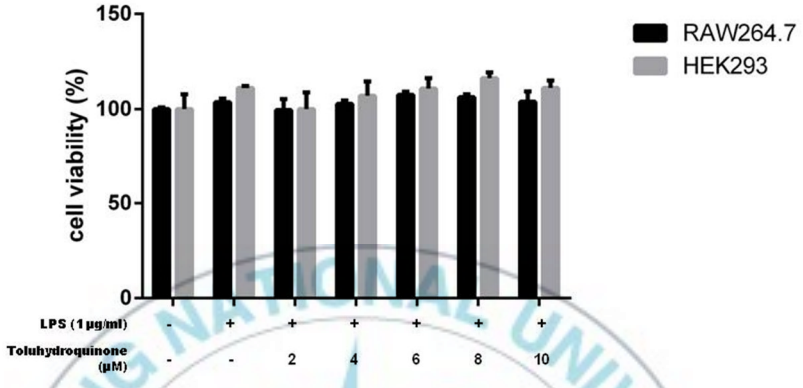

Fig. 2 Effects of toluhydroquinone on the cell viability of RAW264.7 and HEK293 cells. The data were represented the mean ± SD of three independent experiments.

Effects of toluhydroquinone on cells viability

Cell viability was measured by MTT assay. RAW 264.7 macrophage cells and HEK293 human embryonic kidney cells were treated various concentration of toluhydroquinone, then induced with LPS (1 ug/ml). The cytotoxicity of toluhydroquinone was measured by WST-1 solution. Fig. 2 shows no cytotoxicity effects of toluhydroquinone.

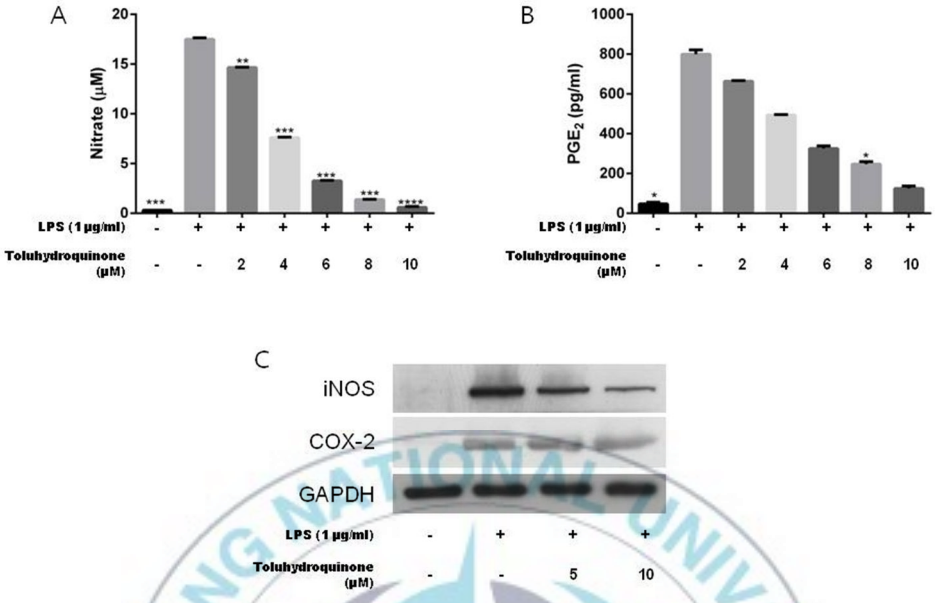

Fig. 3 Effects of toluhydroquinone on **A** NO production, **B** PGE_2 release and **C** iNOS and COX-2 protein expression of LPS-induced RAW264.7 cells. The data were represented the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ significantly different from LPS-induced group.

Toluhydroquinone inhibited NO and PGE_2 production

RAW264.7 cells were pretreated with toluhydroquinone for 2 h and treated with LPS further 24 h. NO production was measured by the amount of NO in culture medium using Griess reagent. As shown in Fig. 3 A, toluhydroquinone inhibited NO production dose dependent-manner. Toluhydroquinone reduced fewer than 50% inhibition of NO production at the concentration of 4 uM, fewer than 5% inhibition of NO production at the concentration of 10 uM. The COX-2 release was measured with PGE_2 production which was measured by ELISA kit. As shown in Fig. 3 B, toluhydroquinone decreased PGE_2 release dose dependent-manner. Toluhydroquinone inhibits PGE_2 release with > 50% the concentration of 6 uM.

Toluhydroquinone decreased iNOS and COX-2 protein expression

The effects of toluhydroquinone on the mechanism of reducing NO and PGE_2 production, iNOS and COX-2 protein expression was measured by western blot analysis. RAW264.7 cells were pretreated with 5 or 10 uM of toluhydroquinone for 2 h and induced with 1 ug/ml of LPS for 18 h. Fig. 3 C is shown that toluhydroquinone reduced iNOS and COX-2 protein expression. Toluhydroquinone affects the reduction of iNOS expression than COX-2 expression.

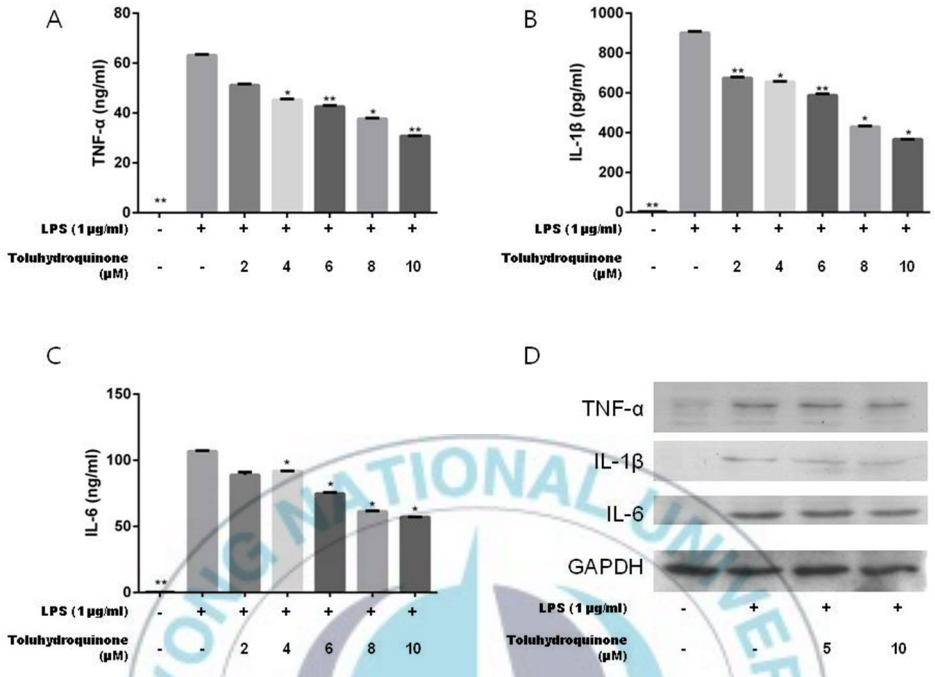

Fig. 4 Effects of toluhydroquinone on pro-inflammatory cytokines production including TNF-α, IL-1β, and IL-6 in LPS-induced RAW264.7 cells. The data were represented the mean ± SD of three independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$ significantly different from LPS-induced group.

Toluhydroquinone reduced pro-inflammatory cytokine production

To measure the pro-inflammatory cytokine, TNF-α, IL-1β, and IL-6 production was examined by ELISA kits and western blot analysis. As shown in Fig. 4 A, toluhydroquinone reduced more than 50% TNF-α expression at the concentration of 10 uM. Also, Fig. 4 B is represented toluhydroquinone inhibited more than 50% IL-1β expression at the concentration of 8 uM. IL-6 expression was shown Fig. 4 C, toluhydroquinone reduced the IL-6 expression with dose dependent-manner. And Fig. 4 D is shown that toluhydroquinone reduced the expression of pro-inflammatory cytokines by western blot analysis. These results represent that toluhydroquinone inhibited the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6.

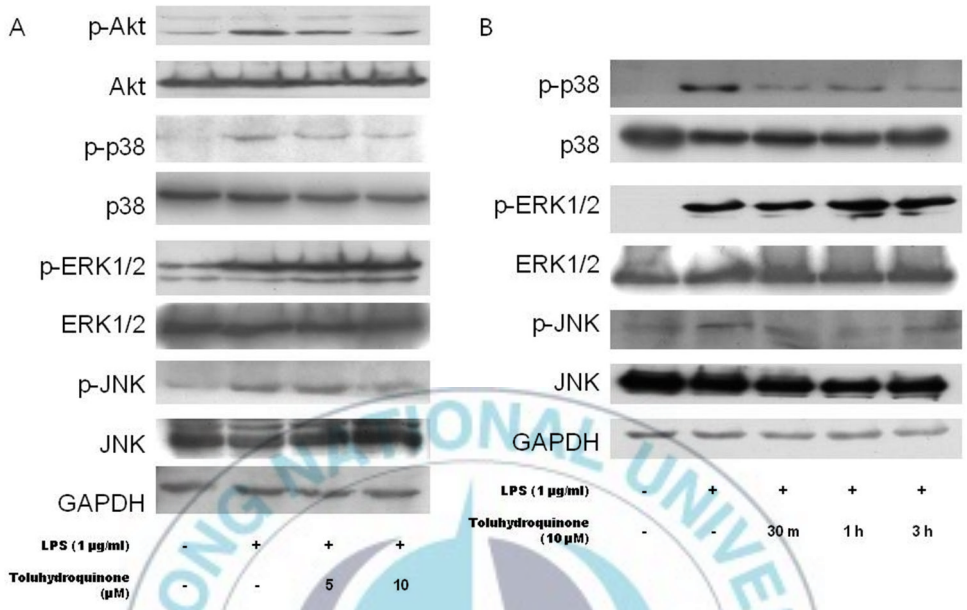

Fig. 5 Effects of toluhydroquinone on expression of phosphorylation of Akt and MAPKs in LPS-stimulated RAW264.7 cells. **A** and **B** were measured by western blot analysis.

Toluhydroquinone suppressed phosphorylation of Akt and MAPKs

MAPKs including p38, ERK 1/2, and JNK regulate immune response. Toluhydroquinone suppressed phosphorylation of Akt and MAPKs, which is presented reducing immune response (Park *et al*., 2013b). RAW264.7 cells were pretreated with 5 or 10 uM of toluhydroquinone for 2 h and treated with 1 ug/ml of LPS for 3 h or as shown and measured by western blot analysis. Fig. 5 A is shown the phosphorylation of Akt and MAPKs including p38, ERK1/2, and JNK at dose dependent-manner. To treat 10 uM of toluhydroquinone, the phosphorylation of p38 is clearly suppressed at dose dependent-manner as well as time dependent-manner (Fig. 5 B). These results indicated toluhydroquinone reduced phosphorylation of MAPKs expression by dose dependent and time dependent.

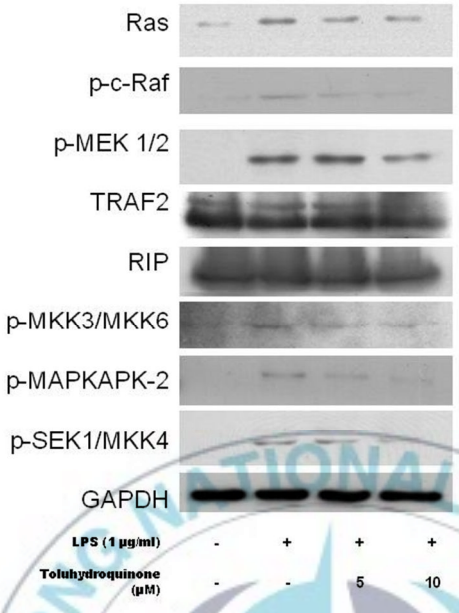

Fig. 6 Effects of toluhydroquinone on phosphorylation of upstream and downstream MAPKs in LPS-induced RAW264.7 cells. These were measured by western blot analysis.

Toluhydroquinone inhibited phosphorylation of upstream and downstream of MAPKs

Fig. 6 is shown upstream and downstream of MAPKs pathway. Ras, c-Raf, MEK 1/2 are upstream of ERK1/2. TRAF2 and RIP are common upstream of p38 and JNK. MKK3/MKK6 is upstream of p38, and MAPKAPK-2 is downstream of p38. SEK1/MKK4 is upstream of JNK. RAW264.7 cells were pretreated with 5 or 10 uM of toluhydroquinone for 2 h and treated with 1 ug/ml of LPS for 3 h or as shown and measured by western blot analysis. Toluhydroquinone did not affect the expression of TRAF2 and RIP by dose dependent manner, but affect the phosphorylation of c-Raf, MEK1/2, MKK3/MKK6, MAPKAPK-2 and SEK1/MKK4 were decreased by dose dependent manner. Also, expression of Ras was decreased by dose dependent manner.

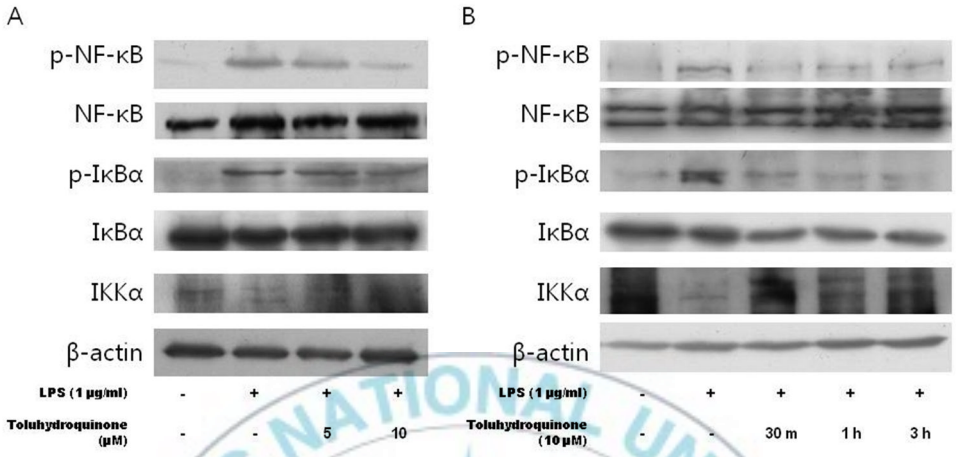

Fig. 7 Effects of toluhydroquinone on of phosphorylation of NF-κB and degradation of IκBα in LPS-induced RAW264.7 cells. **A** and **B** were subjected to western blot analysis.

Toluhydroquinone inhibited phosphorylation of NF-κB and degradation of IκBα

NF-κB regulates inflammatory mediators and is an important transcription factor to produce inflammatory gene (Chow *et al*., 2012). Therefore, toluhydroquinone inhibits the phosphorylation of NF-κB, which is represented reducing NF-κB expression as well as inflammatory gene in LPS-induced RAW264.7 cells. The cells were pretreated with as shown concentration of toluhydroquinone for 2 h and induced with LPS (1 ug/ml) for 3 h or as shown. The protein expression was examined by western blot analysis. Toluhydroquinone inhibited the phosphorylation NF-κB as well as degradation of IκBα by dose dependent and time dependent manner (Fig. 7 A and B). These results represented toluhydroquinone suppressed the phosphorylation of NF-κB and degradation of IκBα by dose dependent and time dependent.

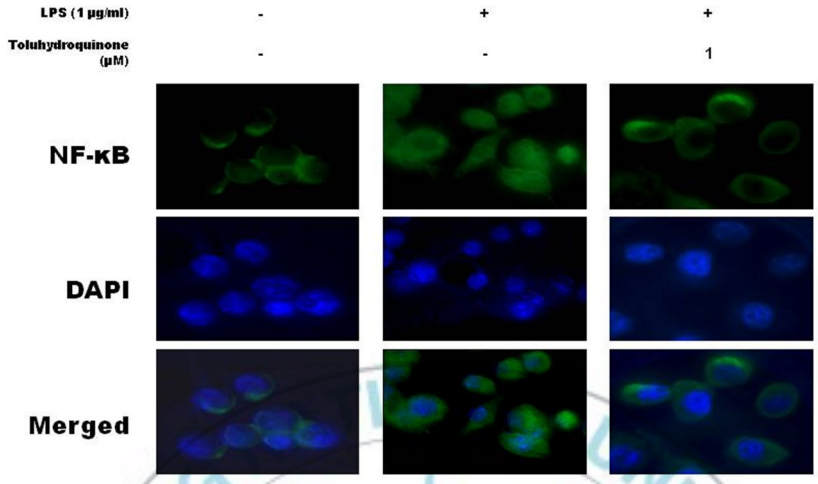

Fig. 8 Effects of toluhydroquinone on NF-κB translocation in LPS-treated RAW264.7 cells, detected by immunofluorescence staining. Cells were stained with DAPI (nuclei, blue) and immunofluorescence antibody (NF-κB, green). Magnification x 1000

Toluhydroquinone prevented NF-κB translocation

With stimulation, NF-κB is translocated from cytosol to the nucleus (Yang *et al*., 2013). Toluhydroquinone prevented NF-κB translocation whether or not, which is examined by immunofluorscence staining. The cells were pre-treated with toluhydroquione (1 uM) for 2 h and further treated with LPS (1 ug/ml) for 12 h. Compared with LPS-induced, toluhydroquinone prevented NF-κB from translocation into nucleus (Fig. 8). These results demonstrated that toluhydroquinone reduced NF-κB translocation.

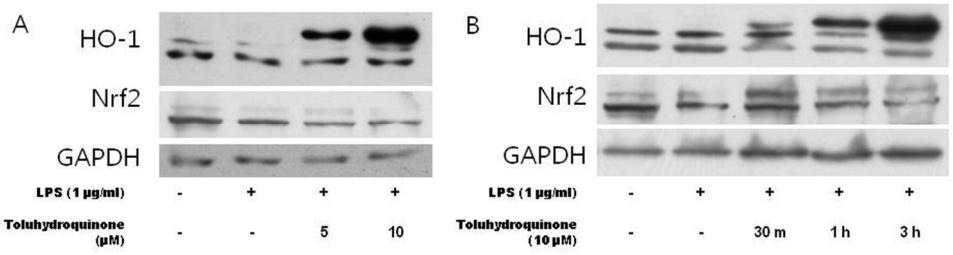

Fig. 9 Effects of toluhydroquinone on HO-1 and Nrf2 protein expression in LPS-stimulated RAW264.7 cells. **A** and **B** were measured to western blot analysis.

Toluhydroquinone induced HO-1 and Nrf2 protein expression

HO-1 regulates inflammatory responses and is reacted to expression of Nrf2 (Kim *et al*., 2012d). Toluhydroquinone induced HO-1 and Nrf2 expression. HO-1 protects the cell from the stimuli and HO-1 reacts transcription factor of Nrf2 (Abuarqoub *et al*., 2006). The cells were pre-treated with 5 or 10 uM of toluhydroquinone for 2 h and stimulated with LPS (1 ug/ml) for 3 h or as shown. Toluhydroquinone produces HO-1 expression (Fig. 9 A and B) with dose dependent as well as time dependent manner. These results indicated that toluhydroquinone stimulated HO-1 protein expression by dose dependent and time dependent.

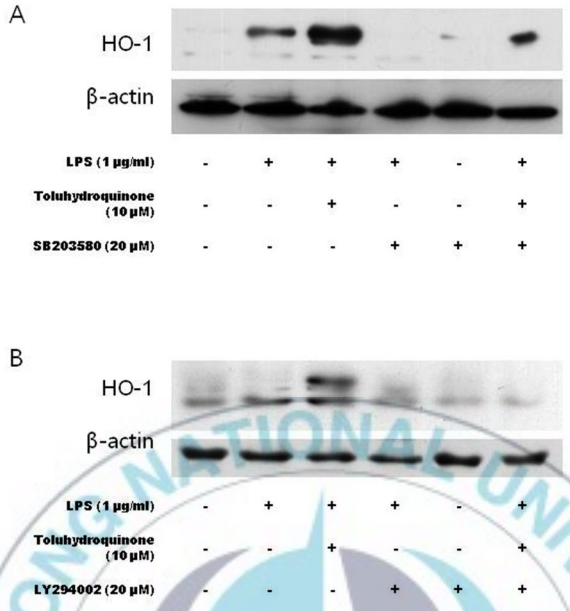

Fig. 10 Effects of toluhydroquinone on HO-1 through p38 and PI3K/Akt pathways in LPS-stimulated RAW264.7 cells. **A** and **B** were measured to western blot analysis.

Toluhydroquinone stimulated HO-1 through p38 and PI3K/Akt pathway

Toluhydroquinone induced HO-1 expression. To prove the role of HO-1-related pathways used the specific inhibitors (SB203580; p38, LY294002; PI3K/Akt). The cells were pre-treated with 20 uM of individual inhibitor for 1 h, with 10 uM of toluhydroquinone for 1 h, and stimulated with LPS (1 ug/ml) for 1 h. Inhibitors of p38 and PI3K/Akt pathways decreased toluhydroquinone-stimulated HO-1 protein expression (Fig. 10 A and B). These results indicated that toluhydroquinone induced HO-1 protein expression through p38 MAPK and Akt pathways.

DISCUSSION

Secondary metabolites, which have known as natural products, produced many endophytic fungus (Cui *et al*., 2012). Toluhydroquinone is one of the secondary metabolites, derived from *Phoma herbarum*. *Phoma herbarum* belong to endophytic fungi *Hypnea saidana*. The bioactivity of toluhydroquinone was reported anti-oxidant effects (Suetrong *et al*., 2009) and the biological effect of toluhydroquinone is rarely known. Therefore, we study the mechanism of toluhydroquinone on anti-inflammatory in LPS-induced RAW264.7 cells.

NO plays an important role in host defense and immune response and is produced by iNOS. NO production was increased, causes many diseases such as carcinogenesis, circulatory shock, and inflammation. Thus, the inhibition of NO production may affect the treatment of inflammatory diseases (Kim *et al*., 2012b; Lee *et al*., 2007). In this study, toluhydroquinone inhibited NO production in dose dependent manner as well as the iNOS protein expression in LPS-induced RAW264.7 cells. These results suggest that toluhydroquinone could be a potential inflammatory therapeutic agent by inhibition of NO production and iNOS expression.

PGE_2 is one of the inflammatory mediators and generated by COX-2, inducible form of cyclooxygenase. PGE_2 is produced by the inflammatory stimuli and high level of COX-2 triggers pro-inflammatory expression (Lee *et al*., 2012; Park *et al*., 2013b). Therefore, reduction of COX-2 and PGE_2 is a key of anti-inflammatory mechanism. In this experiment, toluhydroquinone reduced the production of PGE_2 by concentration dependent manner in LPS-stimulated RAW264.7 cells. But COX-2 protein expression was slightly reduced. Based on these results, toluhydroquinone may affect inhibition of PGE_2 strongly but suppression of COX-2 protein level is slightly effective.

TNF-α, IL-1β, and IL-6 are included in pro-inflammatory cytokines, produced by phosphorylation of NF-κB and degradation of IκBα. Pro-inflammatory cytokines are involved in inflammatory responses. Accordingly, reduction of inflammatory cytokines is an important to estimate treatment of inflammatory diseases (Tang *et al*., 2011). In these results, toluhydroquinone significantly inhibited the expression of TNF-α, IL-1β, and IL-6 in LPS-induced RAW264.7 cells. Therefore, toluhydroquinone could consider as the treatment of inflammatory diseases.

MAPKs and NF-κB signaling are involved to generate inflammatory mediators. MAPKs pathway are related to response the production of NO

and iNOS expression in LPS-stimulated RAW264.7 cells. LPS induced in macrophage cells, phosphorylation of MAPKs pathway is initiated (Kwon *et al*., 2012). Many studies have shown that iNOS and COX-2 expression in LPS-induced are involved phosphorylation of MAPKs (Chun *et al*., 2012). In these studies, toluhydroquinone suppressed phosphorylation of Akt and MAPKs such as p38, ERK1/2, and JNK in LPS-stimulated RAW264.7 cells by dose dependent and time dependent manner. Among MAPKs pathway, phosphorylation of p38 is significantly decreased by dose dependent and time dependent manner. In according to these results, toluhydroquinone may regulate the inhibition of Akt and MAPKs pathway including p38, ERK1/2, and JNK, specifically have an effect on suppression of p38 MAPK pathway.

Ras, c-Raf, and MEK1/2 are upstream of ERK1/2 MAPK pathway. Inhibition of Ras and c-Raf kinase affect to reduce expression of ERK1/2 signaling pathway (Pan *et al*., 2008). These results show that toluhydroquinone reduced expression of ERK 1/2 pathway through Ras and phosphorylation of c-Raf and MEK1/2 by dose dependent manner in LPS-induced RAW264.7 cells. Therefore, toluhydroquinone could regulate to inhibit the phosphorylation of ERK1/2 MAPK pathway.

TRAF2 and RIP is upstream of p38 and JNK MAPK pathway (Yuasa *et al*.,

1998). In these results, the expression of TRAF2 and RIP was no change by dose concentration in LPS-stimulated RAW264.7 cells. SEK1/MKK4 is also upstream of JNK pathway (Wu *et al*., 2008). In these studies, toluhydroquinone suppressed the phosphorylation of SEK1/MKK4 expression by dose dependent manner in LPS-induced RAW264.7 cells. Accordingly, toluhydroquinone did not regulate the protein expression of TRAF2 and RIP. But toluhydroquinone may inhibit the phosphorylation of SEK1/MKK4, which is downstream of TRAF2 and RIP.

MKK3/MKK6 is upstream of p38 MAPK pathway. Phosphorylation of MKK3/MKK6 and p38 mediate downstream pathway through MAPKAPK-2. MAPKAPK-2 controls the many inflammatory genes and thus suppression of phosphorylation of MAPKAPK-2 is effective same as inhibition of phosphorylation of p38 pathway (Yoo *et al*., 2012). In these results, toluhydroquinone suppressed phosphorylation of MKK3/MKK6 and MAPKAPK-2 by dose dependent manner in LPS-induced RAW264.7 cells. These results represent toluhydroquinone leads the inhibition of phosphorylation of p38 MAPK as well as MKK3/MKK6 and MAPKAPK-2.

MAPKs pathway is considered to activate NF-κB. Accordingly, inhibition of MAPKs has an effect on inhibition of NF-κB activity as well (Rehman *et al*.,

2012). NF-κB is a major transcription factor in cellular responses, expresses pro-inflammatory cytokines. The promoter regions of iNOS and COX-2, and inflammatory genes are located in NF-κB (Oh *et al*., 2012). Therefore, inhibition of NF-κB is a crucial factor to treat inflammatory mediated diseases. In these experiments, toluhydroquinone decreased phosphorylation of NF-κB and degradation of IκBα by dose dependent as well as time dependent manner in LPS-induced RAW264.7 cells. Also, translocation of NF-κB is demonstrated by immunofluorescence staining in low concentration of toluhydroquinone. According to these results, toluhydroquinone react to inhibit phosphorylation of NF-κB, degradation of IκBα and as well as translocation of NF-κB into nucleus.

HO-1 regulates the degradation of pro-inflammatory free-heme and uses CO and bilirubin to produce anti-inflammatory, anti-oxidant, and anti-apoptotic (Kim *et al*., 2012d). Nrf2 is an upstream of HO-1 expression, which is expressed when Nrf2 translocates into nucleus (Jin *et al*., 2012). Hence, increasing HO-1 expression is the key of inflammatory related diseases treatments. In these results, toluhydroquinone induced the expression of HO-1 by dose dependent and time dependent in LPS-stimulated RAW264.7 cells. And p38 and PI3K/Akt pathways are related in toluhydroquinone-induced

HO-1 protein expression in macrophages.

In conclusion, our study demonstrated that the mechanism of toluhydroquinone, which is secondary metabolites from *Phoma herbarum*, on anti-inflammatory in LPS-induced RAW264.7 cells. Toluhydroquione inhibited inflammatory mediators including NO, PGE_2, TNF-α, IL-1β, and IL-6 production as well as iNOS, COX-2, TNF-α, IL-1β, and IL-6 protein expression. Also, toluhydroquinone suppressed phosphorylation of upstream and downstream MAPKs pathway such as p38, ERK1/2, and JNK. Furthermore, toluhydroquinone reduced the phosphorylation of NF-κB, translocation of NF-κB, and degradation of IκBα. Moreover, toluhydroquinone induced HO-1 expression through p38 and PI3K/Akt pathways. Thus, these results supports that toluhydroquinone may be a potential anti-inflammatory reagent after further *in vivo* studies.

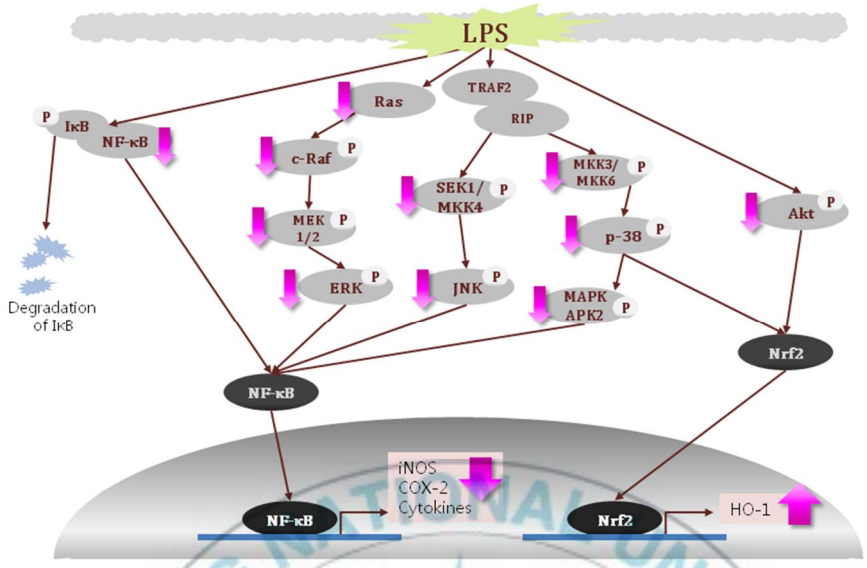

Fig. 10 Possible anti-inflammatory signaling pathway by toluhydroquinone in LPS-induced RAW264.7 cells

국문 초록

무척추동물, 조류, 미생물 등 다양한 해양 생물들이 이차 대사산물을 생성하며 이들 중 몇몇의 이차 대사산물은 여러 질병의 화학치료제로 고려되고 있다. Toluhydroquinone은 *Hypnea saidana*에 공생하는 해양 곰팡이 *Phoma herbarum*이 생산한 이차 대사산물에 의해 생성된 것으로 그 활성은 알려진 바가 적다. 따라서 toluhydroquinone의 항염증 매커니즘을 알아보기 위해 RAW264.7 cell에 LPS를 처리하여 염증 반응을 유도하였을 때 염증 관련 인자들의 생성 억제를 알아보았다.

Toluhydroquinone을 처리하였을 때, NO생성, PGE_2, 염증 조절 단백질인 iNOS, COX-2 발현이 억제되며, TNF-α, IL-1β, IL-6 등 염증 유발 인자 cytokine을 감소시킨다. Toluhydroquinone은 NF-κB와, Akt, MAPKs pathway에 포함된 ERK, JNK, p38의 인산화를 시간과 농도에 비례하여 억제시킨다. 또한, toluhydroquinone은 p38과 PI3K/Akt pathway를 통해 스트레스 유도 효소인 HO-1 발현을 증가시켜 항염증 효과를 상승시킨다. 이러한 결과는 toluhydroquinone이 RAW264.7 cell에 LPS를 유도했을 때 NF-κB, MAPK pathway를 통해 염증 관련 인자의 발현을 감소시키고 항염증 인자의 발현을 상승시켜 항염증 메커니즘에 효과가 있다고 제안할 수 있다.

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