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Immunomodulatory Activities of Medicinal Mushroom *Grifola frondosa* Extract and Its Bioactive Constituent

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Abstract: *Grifola frondosa* (GF), a high value medicinal mushroom in China and Japan, is popularly consumed as traditional medicines and health foods, especially for enhancing immune functions. In this study, our aim was to examine the immunomodulatory activities of GF and its bioactive compound ergosterol peroxide (EPO) in lipopolysaccharide (LPS)-induced human monocytic (THP-1) cells. At low concentrations, EPO but not other extracts showed a full protection against LPS-induced cell toxicity. EPO significantly blocked MyD88 and VCAM-1 expression, and cytokine (IL-1β, IL-6 and TNF-α) production in LPS-stimulated cells. It also effectively inhibited NF-κB activation, which was further confirmed with siRNA treatment. These results conclude that EPO may play an important role in the immunomodulatory activity of GF through inhibiting the production of pro-inflammatory mediators and activation of NF-κB signaling pathway.

Keywords: *Grifola frondosa*; Medicinal Mushroom; Ergosterol Peroxide; Immunomodulatory Activity; Pro-Inflammatory Mediators; THP-1 Cells.

Introduction

Grifola frondosa (Dicks.) Gray (GF), a valuable medicinal mushroom, is popularly consumed as traditional medicines and health foods in China and Japan. Studies have shown that *G. frondosa* possessed numerous medicinal properties, including anticancer (Kodama *et al.*, 2005; Frank *et al.*, 2006), anti-inflammatory (Lee *et al.*, 2010), immunostimulatory

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(Kodama *et al.*, 2005), anti-hypertensive (Talpur *et al.*, 2002) and antidiabetic (Manohar *et al.*, 2002) activities. Its D-fraction effectively inhibited inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in LPS-induced Raw 264.7 cells (Sanzen *et al.*, 2001). Despite the many biological activities of *G. frondosa* being reported, bioactive compounds contributing to its various bioactivities remain largely unknown.

Inflammation is one of the first responses of the immune system to infection and irritation. Chronic inflammation is known to be an important causing factor leading to various chronic diseases including atherosclerosis, diabetes and cancer (Manabe, 2011; Essafi-Benkhadir *et al.*, 2012). Although many drugs have been developed for treating these diseases, side effects remain a critical issue for many of the clinically used drugs. With the increasing interest on the use of edible and medicinal mushrooms as natural sources for preventing and curing chronic diseases, the discovery and identification of novel immunomodulatory agents from mushrooms have become an important topic of research worldwide.

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is recognized by monocytes and macrophages of the innate immune system (Youn *et al.*, 2006; Wu *et al.*, 2007). MyD88 is an immediate adaptor molecule recruited by activated TLR4, and plays an important role in triggering the activation of signaling cascades, including IRAK-1, IRAK-4, TRAF-6, and NF-κB (Rhee and Hwang, 2000; Takeda and Akira, 2005). Nuclear factor-kappa B (NF-κB), an important transcription factor, is known to regulate the expression of genes critical for a variety of biological processes, including immune responses, inflammatory reactions and apoptosis (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). In response to inflammatory stimuli LPS, NF-κB is activated and translocated into nucleus where it up-regulates a number of inflammation-related genes such as cytokines (IL-1, IL-2, IL-6, IL-8 and TNF-α), cell adhesion molecules (E-selectin, ICAM-1 and VCAM-1), iNOS and COX-2 (Yamamoto and Gaynor, 2001; Fong *et al.*, 2008). Hence, targeting these pro-inflammatory mediators might provide an interesting and novel way to prevent and treat various inflammatory-related diseases.

In this study, our aims were: (i) to evaluate the effects of GF extracts and their bioactive compound ergosterol peroxide (EPO) on cell viability in LPS-induced human monocytic (THP-1) cells, and (ii) to examine the effects of EPO on MyD88, VCAM-1 and NF-κB expression, and cytokine (IL-1β, IL-6 and TNF-α) production.

Materials and Methods

Chemicals

Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), penicillin, streptomycin, anti-β-actin and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from GIBCO BRL (Gaithersburg, MD, USA). Anti-VCAM-1 and anti-NF-κB p65 anti-bodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-MyD88 anti-body was purchased from Cell Signaling

Technology Inc. (Danvers, MA, USA). The anti-mouse IgG antibody was obtained from Promega (Madison, WI, USA). All other chemicals used were of analytical grade.

G. frondosa Materials and Extract Preparation

The fruiting bodies of cultured *G. frondosa* were obtained from Kang Jian Biotech Corp., Ltd. (Nantou Hsien, Taiwan). They were ground to powdered form and collected in an airtight plastic bag until use.

To prepare the aqueous extracts, 100 g of *G. frondosa* powder was extracted with 1 L of boiling water for 1 h. For ethanol extract, 100 g of *G. frondosa* powder was extracted with 1 L of 95% ethanol at room temperature for six days. After filtering with filter paper (Advantec No. 1, Advantec Co. Ltd., Tokyo, Japan), the filtrate was concentrated and lyophilized. The dried aqueous (GF-H) and ethanol (GF-E) extracts of *G. frondosa* were collected and stored at 4°C until use.

To prepare the GF-polysaccharides, 100 g of dried GF powder was extracted with 1 L of boiling water for 1 h. After allowing the samples to cool to room temperature, the solid particles were removed by centrifugation at 5000 g for 30 min, followed by filtering through filter paper (Advantec No. 1). Two volumes of 95% ethanol to one volume of the filtrate were then added to precipitate the polysaccharides, which were then concentrated and lyophilized. The dried polysaccharides (GF-P) were collected and stored at 4°C until use.

Ergosterol Peroxide (EPO) Preparation

Three kg of GF powder were extracted with n-hexane (10 L) under ambient temperature for two weeks twice consecutively. The filtrates obtained from two separate extractions were combined and concentrated to obtain the hexane extract 128 g, which was then performed on a Si gel (1.2 kg) column chromatography with gradient solvent system of EtOAc-hexane from 0–40%. Ergosterol (5.80 g) was obtained with the elution of 10–15% EtOAc-hexane, while ergosterol peroxide (225 mg) was obtained with 20% of the same solvent system. These products were further purified by crystallization in the same solvent system. The structure and purity of EPO were confirmed with mass spectrometry and nuclear magnetic resonance spectroscopy (Fig. 1). Details of chromatographic spectra of EPO have been reported previously (Krzyczkowski *et al.*, 2009).

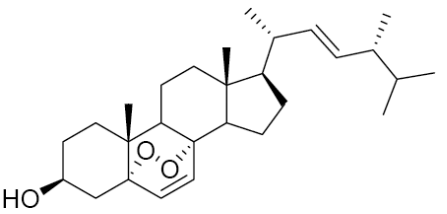


Figure 1. Structure of ergosterol peroxide.

Cell Culture

THP-1, a promonocytic cell line, was obtained from the American Type Culture Collection (ATCC No. TIB-202; Manassas, VA, USA). Cells were grown in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Cell Viability Assay

Cell viability was examined by MTT assay. In brief, THP-1 cells were cultured at 1×10^5 cells per well in 96-well plates containing 100 μl of RPMI-1640 medium. After an overnight incubation, cells were treated with 1% DMSO (control) or LPS (1 μg/ml) alone or LPS (1 μg/ml) plus various concentrations of GF-H (10, 50 and 100 μg/ml), GF-P (5, 10, 30 and 50 μg/ml), GF-E (10, 50 and 100 μg/ml) or EPO (1, 5, 10, 20 and 30 μM), followed by incubating the plates for 48 h. Cells were washed once before adding 50 μl of FBS-free medium containing 5 mg/ml MTT. After incubating at 37°C for 4 h, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO. The optical density was measured at 550 nm.

Cytoplasmic Extract Preparation

Cytoplasmic extracts were prepared as described previously (Wu *et al.*, 2007). In brief, after treating the cells without and with LPS (1 μg/ml) alone or LPS plus EPO (5, 10 and 20 μM), they were washed once with PBS and then lysed in ice-cold buffer I (10 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.4 M sodium chloride, 1 mM PMSF) containing 2 μg/ml leupeptin, 2 μg/ml aprotonin and 0.5 mg/ml benzamidine. After incubating the cells on ice for 20 min, 10 μl of 10% Nonidet P-40 was added. Lysates were centrifuged at 10,000 g at 4°C for 10 min to obtain the supernatant, which was collected as the cytoplasmic extract and was stored at −80°C until use. Protein concentrations in these extracts were determined by Bio-Rad reagents (Bio-RAD Laboratories, Hercules, CA, USA).

Nuclear Extract Preparation

Nuclear protein extracts were prepared as described previously (Wu *et al.*, 2007). Briefly, after PBS washing, cells were scraped off the plates in 0.6 ml ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, and 2 μg/ml each of aprotinin, pepstatin and leupeptin). After centrifugation at 300 g at 4°C for 10 min, cells were suspended in buffer B (80 μl of 0.1% Triton X-100 in buffer A) and then left on ice for 10 min, followed by centrifugation at 12000 g at 4°C for 10 min. The nuclear pellets collected were resuspended in 70 μl ice-cold buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 1 mM DTT, 0.2 mM EDTA, 1 mM PMSF, 25% glycerol, and 2 μg/ml each of aprotinin, pepstatin and leupeptin), and then incubated at 4°C for 30 min,

followed by centrifugation at 15,000 g at 4°C for 30 min. The resulting supernatant was used as the nuclear extract and was stored at −80°C until use. Protein concentrations in these extracts were determined by Bio-Rad reagents.

Western Blot Analysis

Western blot analysis of anti-MyD88, anti-VCAM-1 and anti-NF-κB was carried out by employing the respective antibodies. In brief, cytoplasmic proteins (50 μg/lane) were loaded onto SDS-polyacrylamide gels and electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After inhibiting the nonspecific binding sites with 5% (w/v) skim milk in 0.1% (v/v) Tween 20 containing PBS (PBST) for 1 h at room temperature, the membrane was incubated with the specific primary antibodies for 1 h at room temperature. Antibody recognition was detected with anti-mouse IgG or anti-rabbit antibody linked to the horseradish peroxidase. Antibody-bound proteins were detected by the ECL western blotting analysis system (Amersham, Aylesbury, UK). Both β-actin (cytosol fractions) and LAM B1 (nuclear fractions) were used as the positive control. Relative protein expression was quantified densitometrically with an AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA, USA) using AlphaEaseFC software, and calculated according to the β-actin or LAM B1 reference band.

Measurement of Cytokines

Cells were induced with 1 μg/ml LPS in the absence or presence of EPO at 10 and 20 μM for 48 h. The concentrations of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) in culture supernatants were measured using ELISA kits and performed according to the manufacturer's instructions (RayBiotech Inc., Norcross, GA, USA).

Measurement of NF-κB Activation

Cells were treated with 1% DMSO (control), LPS (1 μg/ml) alone, LPS (1 μg/ml) plus various concentrations of EPO (10 and 20 μM), followed by incubating the 96-well plates for 48 h. The nuclear NF-κB activation was measured by a NF-κB p65/RelA transcription factor assay kit according to the manufacturer's instruction (Cayman Chem Co., Ellsworth Rd, MI, USA).

NF-κB siRNA Transfection

NF-κB p65 siRNA was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). A non-specific siRNA was used as a negative control (control group siRNA). In brief, 2×10^5 cells were plated on 10 cm dishes and left to grow overnight, followed by transfecting with NF-κB p65 siRNA using transfection reagent (Qiagen, Velencia, CA, USA) for 48 h according to the manufacturer's instructions. The final concentration of NF-κB p65 siRNA was 100 nM. To conform the efficacy of NF-κB p65 siRNA, western blotting assay was conducted using anti-NF-κB antibody.

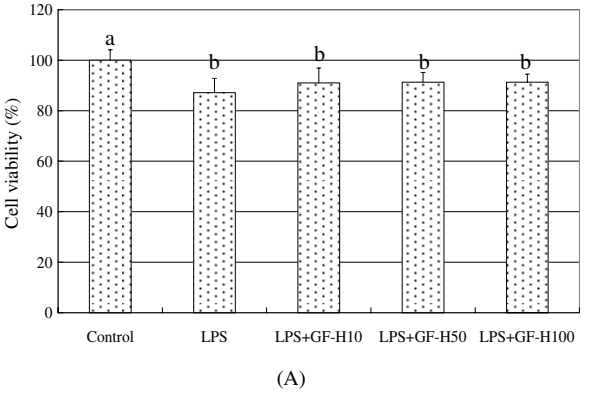
Statistical Analysis

Data were presented as mean±standard deviation (S.D.) of three independent experiments. Values were evaluated by one-way ANOVA, followed by Duncan's multiple range tests using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences were considered significant when p value was < 0.05.

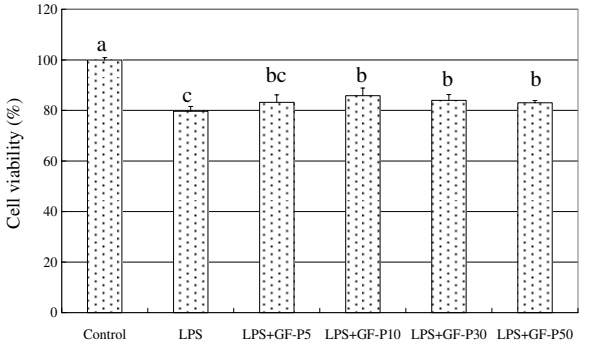
Results

Effects of G. frondosa Extracts, Polysaccharides and EPO on Cell Viability

Figure 2 shows the effects of *G. frondosa* extracts, polysaccharides and EPO on LPS-induced THP-1 cell viability. Results showed that GF-H and GF-polysaccharides at

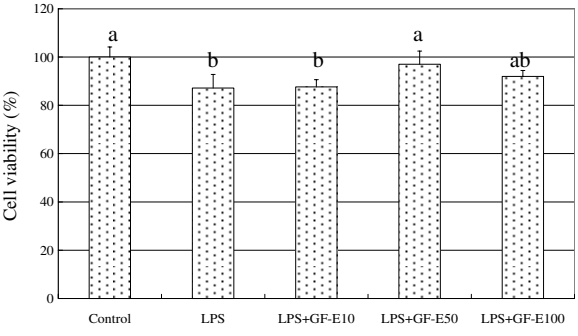


(A)

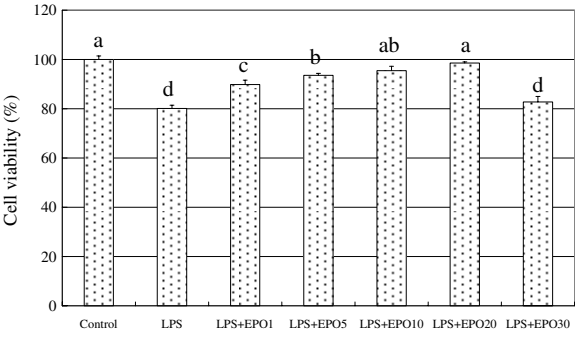


(B)

Figure 2. Effects of *G. frondosa* extracts and bioactive compound on THP-1 cell viability. Cells were stimulated with LPS (1 μg/ml) for 48 h in the absence or presence of test samples at various concentrations. (A) GF-H; (B) GF-P; (C) GF-E; and (D) EPO. Cell viability was analyzed by MTT assay. Data are presented as mean ± S.D. of three independent experiments. The bar having the same letter within treatment was not significantly different at $p < 0.05$ as analyzed by Duncan's multiple range tests.



(C)



(D)

Figure 2. (*Continued*)

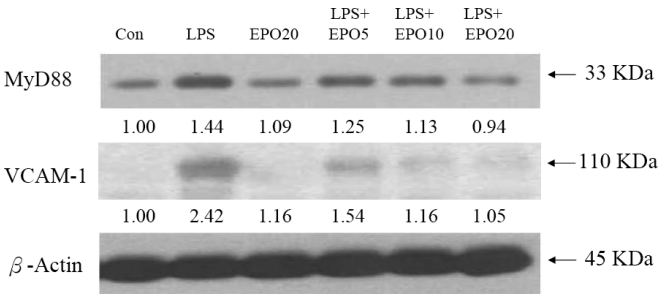
100 μg/ml showed only 80% protection against LPS-induced cell toxicity, whereas a complete protection was noted on GF-E at 50 μg/ml, and EPO at 10–20 μM. EPO at 1–20 μM showed a dose-dependent increase in protecting THP-1 cells against LPS-induced toxicity. Therefore, EPO was selected for further studies of immunomodulatory properties in THP-1 cells.

EPO Influenced MyD88 and VCAM-1 Expression

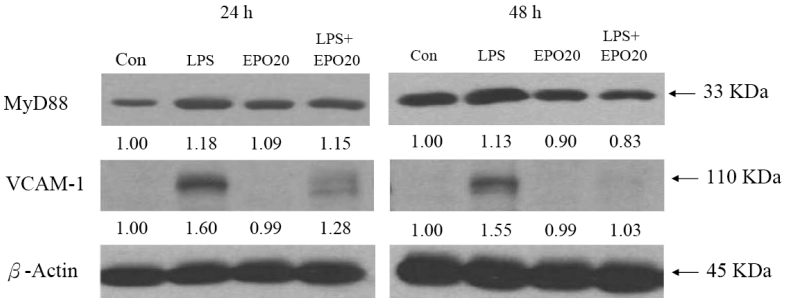
Results showed that LPS treatment led to a marked up-regulation of MyD88 and VCAM-1 expression as compared to the un-treated cells; however they were down-regulated after EPO only or 1 μg/ml LPS plus 20 μM EPO treatments for 24 and 48 h (Figs. 3A and 3B). After 48 h treatment, EPO significantly inhibited LPS-stimulated expression of MyD88 and VCAM-1 in a concentration dependent fashion (Fig. 3A).

EPO Suppressed the LPS-Induced Cytokine Production

To determine the effects of EPO on the production of proinflammatory cytokines (IL-1β, IL-6 and TNF-α), the cytokine levels presence in the media after treatment for 48 h were



(A) Dose-effect (48 h)



(B) Time-effect

Figure 3. Effects of EPO on MyD88 and VCAM-1 expression in LPS-induced THP-1 cells. (A) Cells were stimulated with 0.1% DMSO (control), 1 μg/ml LPS only, 20 μM EPO only, and 1 μg/ml LPS plus 5, 10 and 20 μM EPO for 48 h, and (B) cells were treated with 0.1% DMSO (control), 1 μg/ml LPS only, 20 μM EPO only, and 1 μg/ml LPS plus 20 μM EPO for 24 and 48 h. Total cell extracts were prepared and analyzed by western blot assay. β-Actin was used as loading control.

measured by ELISA kits. Results showed that compared with untreated cells, LPS-treated cells demonstrated significant increase in IL-1β, IL-6 and TNF-α levels; however their production were suppressed after EPO treatment (Figs. 4A–4C).

EPO Inhibited LPS-Induced Activation of Nuclear NF-κB

NF-κB is an important transcription factor for downstream pro-inflammatory mediators. The suppressive effect on its activation in nucleus by EPO was examined using western blotting assay and NF-κB p65/RelA transcription factor assay kit. Results showed that in unstimulated cells, low basal activity of NF-κB was detected and was significantly enhanced after LPS challenge (Fig. 5A). EPO-treated cells obviously down-regulated the nuclear NF-κB activation as demonstrated by a reduction in the intensity of the nuclear NF-κB bands. Compared with LPS-treated group, EPO at 10 μM and 20 μM significantly suppressed the nuclear NF-κB expression at 24 h and 48 h (Figs. 5A and 5B). Figure 5C shows that LPS significantly increased the NF-κB p65 activity, however a dose-dependent decrease in NF-κB p65 activity was noted on EPO-treated groups.

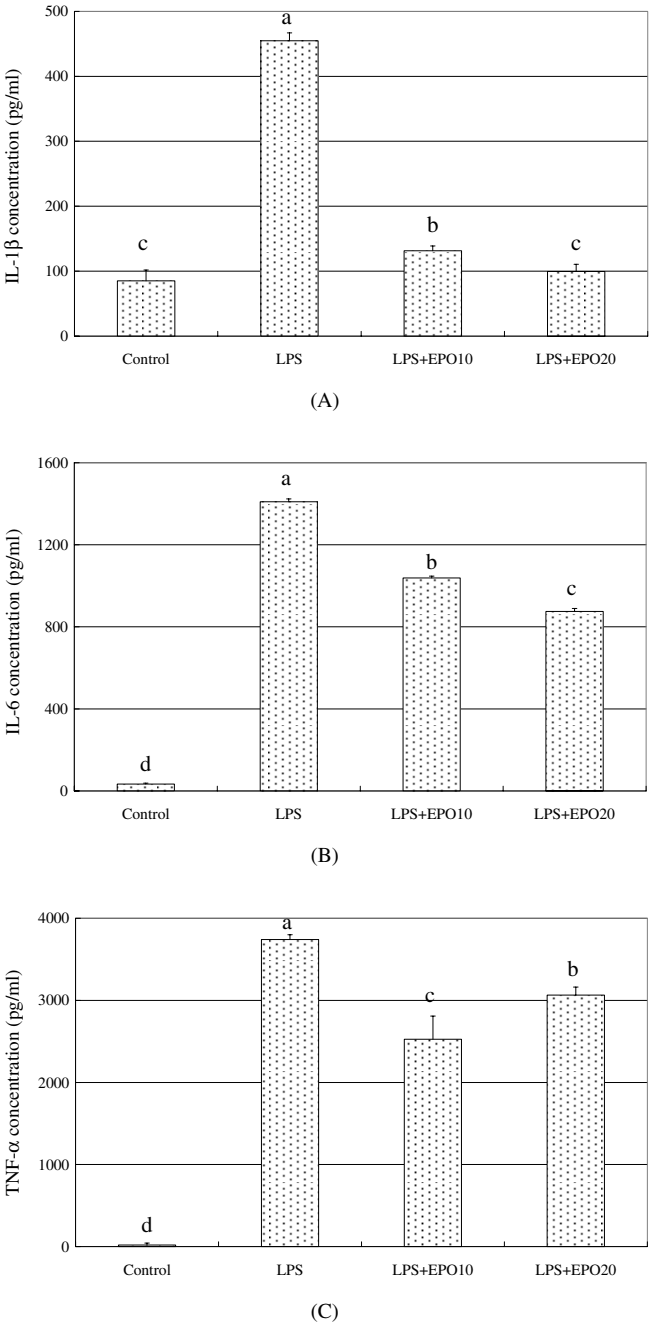
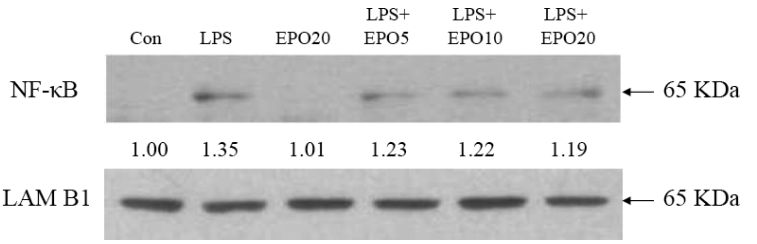
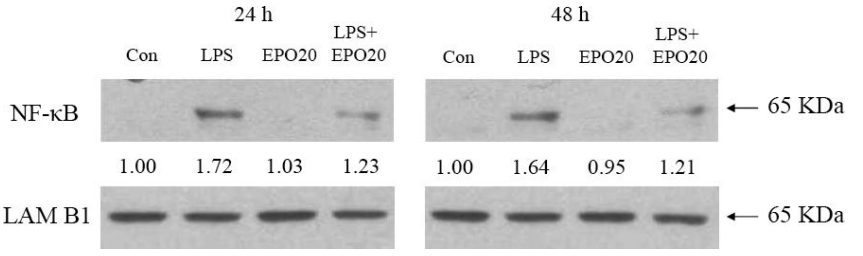


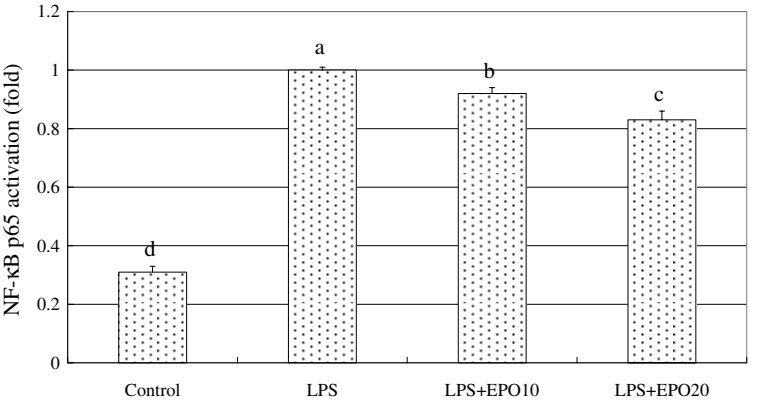
Figure 4. Effects of EPO on IL-1β, IL-6 and TNF-α production in LPS-induced THP-1 cells. Cells were treated with LPS (1 μg/ml) for 48 h in the absence or presence of EPO at 10 and 20 μM. IL-1β, IL-6 and TNF-α concentrations were determined as described in methods and materials section. Data represent mean $\pm$ S.D. of three independent experiments. The bar having the same letter was not significantly different at $p < 0.05$ as analyzed by Duncan's multiple range tests.



(A) Dose-effect (48 h)



(B) Time-effect



(C) NF-κB p65 activation (48 h)

Figure 5. Effects of EPO on nuclear NF-κB activation in LPS-induced THP-1 cells. (A) Cells were treated with 0.1% DMSO (control), 1 μg/ml LPS only, 20 μM EPO only, and 1 μg/ml LPS plus 5, 10 and 20 μM EPO for 48 h; (B) cells were treated with 0.1% DMSO (control), 1 μg/ml LPS only, 20 μM EPO only, and 1 μg/ml LPS plus 20 μM EPO for 24 and 48 h. After treatments, the nuclear extracts of cells were prepared and subjected to western blotting assay where Lam B1 was used as a loading control. (C) Cells were treated with LPS (1 μg/ml) for 48 h in the absence or presence of EPO at 10 and 20 μM, the NF-κB p65 activation was measured by NF-κB p65/RelA transcription factor assay kit. Data represent the mean $\pm$ S.D. of three independent experiments. The bar having the same letter was not significantly different at $p < 0.05$ as analyzed by Duncan's multiple range tests.

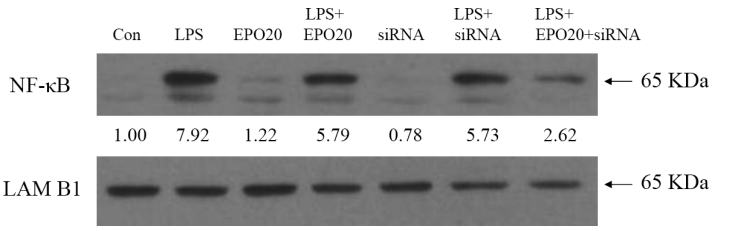


Figure 6. Effects of EPO and siRNA on NF-κB (nucleus) expression in LPS-induced THP-1 cells. Cells were stimulated with LPS (1 μg/ml) for 48 h in the presence or absence of EPO (20 μM) and siRNA. The nuclear extracts were prepared and analyzed by western blot assay. Lam B1 was used as a loading control.

Figure 6 shows that expression of NF-κB p65 was inhibited by siRNA in LPS-activated THP-1 cells. Co-administration of siRNA and EPO further suppressed the NF-κB expression at 48 h.

Discussion

This study demonstrated that LPS-induced THP-1 cell toxicity was completely protected by 10–20 μM EPO and 50 μg/ml GF-E, but not GF-H and GF-polysaccharides at 100 μg/ml. EPO showed significant suppressive effects on MyD88 and VCAM-1 expression, and cytokine (IL-1β, IL-6 and TNF-α) production in LPS-stimulated THP-1 cells. In addition, EPO also significantly blocked the activation of NF-κB.

Human monocytic cells are known to play a vital role in LPS-induced inflammatory responses by the production of proinflammatory mediators (Wu *et al.*, 2007). Quercetin and luteolin were reported to inhibit the LPS-induced cytokine production in macrophages (Xagorari *et al.*, 2002; Cho *et al.*, 2003). In this study, we showed that EPO significantly inhibited the LPS-induced production of pro-inflammatory mediators such as IL-1β, IL-6 and TNF-α in THP-1 cells; such anti-inflammatory responses were closely associated with its inhibitory effect on LPS-induced NF-κB activation. This observation supports the suppressive effects of EPO on the downstream signaling pathways.

Numerous surface molecules such as pattern recognition receptors TLR4 and their adaptor molecules such as TRIF and MyD88 are identified as a critical regulatory receptor of inflammatory process (O'Neill, 2003). LPS triggers the activation of TLR4 and its adapter proteins such as TRIF and MyD88, and allow the activation of macrophages to produce various inflammatory products that are linked to transcriptional activation of NF-κB. Targeted blockage of these signaling pathways could therefore be considered as a possible therapeutic approach to develop anti-inflammatory agents for treating inflammation-mediated chronic diseases (Garcia-Lafuente *et al.*, 2009). Under our conditions, EPO displayed strong inhibitory effects on MyD88 and VCAM-1 expression in response to LPS-stimulation. Furthermore, EPO also significantly suppressed NF-κB activation, suggesting that EPO is able to suppress the translocation of NF-κB from cytosol to nucleus, and subsequently triggered the down-regulation of the inflammatory related genes. Since

EPO strongly blocked NF-κB translocation, this suggests that this compound could be a major active principle of anti-inflammation in *G. frondosa* extracts.

VCAM-1 is an inducible molecule that has been shown to mediate endothelial adhesion of monocytes and T lymphocytes (Springer, 1990), the principal leukocyte subsets populating atherosclerotic lesions (Ross, 1986). Its expression has been demonstrated to associate with macrophages in rheumatoid synovium (Koch *et al.*, 1991). In cancer patients, tumor cells were noted to escape T-cell immunity by over expressing VCAM-1, and develop into metastatic cells (Wu, 2007). Hence, the suppression of VCAM-1 expression could be an important target in the prevention of cancer and inflammatory related diseases. Studies have shown that polyphenols from olive oil and red wine were effective in suppressing VCAM-1 expression in human umbilical vein endothelial cells (Carluccio *et al.*, 2003). In this study, EPO showed strong inhibitory effect on VCAM-1 expression in LPS-stimulated THP-1 cells, suggesting that EPO is a potent agent in inhibiting VCAM-1 expression, and could be a promising agent for preventing chronic diseases.

EPO inhibited the activation of NF-κB, which is an important transcription factor for iNOS (Gao *et al.*, 1997; Xie *et al.*, 1994). Although the effects of EPO on COX-2 and iNOS expression, and NO production were not measured, because NF-κB is involved in the activation of several downstream inflammatory genes, the inhibition of EPO on NF-κB activation would likely to lead to the down-regulation of the production of an array of inflammatory mediators. In this study, EPO significantly down-regulated the nuclear NF-κB expression, and the NF-κB p65 activation as compared with cells treated with LPS only. The use of siRNA also showed significant down-regulation of NF-κB p65 expression. These results further confirmed that the anti-inflammatory activity of GF is derived from EPO.

In conclusion, this study has demonstrated that EPO provides a better protection than GF-H, GF-E and GF-polysaccharides against LPS-induced cytotoxicity. EPO showed good anti-inflammatory activity as demonstrated by its inhibitory effects on cytokine (IL-1β, IL-6 and TNF-α) production, VCAM-1 expression and NF-κB activation. This is the first study to show that the immunomodulatory activity of GF is in part through EPO, of which the mechanism(s) of action was through inhibiting pro-inflammatory mediator production via suppressing activation of NF-κB signaling pathway.

Acknowledgments

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