

Ganoderma lucidum mycelia enhance innate immunity by activating NF- κ B

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Received 20 December 2004; received in revised form 3 June 2005; accepted 6 August 2005

Available online 15 September 2005

Abstract

Ganoderma lucidum is a popular medicinal mushroom in China and Japan for its immunomodulatory and antitumor effects. The goal of this research is to investigate the effect of dried mycelia of *Ganoderma lucidum* produced by submerged cultivation on the enhancement of innate immune response. We found that *Ganoderma lucidum* mycelia (0.2–1.6 mg/ml) stimulated TNF- α and IL-6 production after 8 h treatment in human whole blood. IFN- γ release from human whole blood was also enhanced after 3 day-culture with *Ganoderma lucidum* mycelia (0.2–1.0 mg/ml). However, *Ganoderma lucidum* mycelia did not potentiate nitric oxide production in RAW264.7 cells. To better understand the possible immuno-enhancement mechanisms involved, we focused on nuclear factor (NF)- κ B activation. Electrophoretic mobility shift assay revealed that the *Ganoderma lucidum* mycelia (1.6 mg/ml) activated κ B DNA binding activity in RAW264.7 cells. These results provide supporting evidences for the immunomodulatory effect of *Ganoderma lucidum* mycelia.

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Keywords: *Ganoderma lucidum*; TNF- α ; IL-6; IFN- γ ; NF- κ B

1. Introduction

Ganoderma lucidum is a medicinal mushroom which has been widely used in China (named Ling Zhi) and Japan (named Reishi, Mannentake) for hundreds of years for the immunomodulating and anti-tumor effects (Yun, 1999; Shiao, 2003). The activities of hypertension, hyperglycemia, hepatitis, chronic bronchitis, bronchial asthma, liver protection and others have also been demonstrated from the fruiting bodies and cultured mycelia of *Ganoderma lucidum* (Yun, 1999; Lu et al., 2002). Many active substances, in particular polysaccharides, with

immunity enhancement effects have been isolated from the water extract of *Ganoderma lucidum*. Among the multiple polysaccharides, active β -D-glucans are responsible for the anti-tumor effect (Miyazaki and Nishijima, 1981; Usui et al., 1981; Sone et al., 1985; Kishida et al., 1988; Slivova et al., 2004). Recent studies also showed that the alcohol extract or the triterpene fraction of *Ganoderma lucidum* possessed anti-tumor effect, which seemed to be related to the cytotoxic activity against tumor cells directly (Lin and Zhang, 2004).

The induction of cytokine synthesis is one of the methods to evaluate augmentation activity of innate immunity. Cytokines are intercellular signaling proteins released by both immune and non-immune cells. They play important roles in controlling homeostasis of the whole organism by the induction of cell differentiation, proliferation and apoptosis, as well as defense functions such as immune responses and inflammatory reactions. In the case of immunomodulatory activity of *Ganoderma lucidum*, special attentions have been paid to the induction of TNF- α , IL-6 and IFN- γ (Wang et al., 1997; Berovic et al., 2003). TNF- α , IL-6 and IFN- γ known as proinflammatory cytokines, modulate the acute phase response that involves potent systemic and local effects. The release of proinflammatory cytokines is

Abbreviations: DMEM, Dulbecco's modified eagle's medium; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GL, the commercialized spray dried *Ganoderma lucidum* mycelia produced by Taiwan Sugar Co.; IFN- γ , interferon- γ ; I- κ B, inhibitors of κ B; IL-6, interleukin-6; LPS, lipopolysaccharide; MAPK, p38 mitogen-activated protein kinase; NF- κ B, nuclear transcription factor- κ B; NO, nitric oxide; NOS, nitric oxide synthase; PKC, protein kinase C; SPG, the triple helical conformer of β -glucan; SPG-OH, the single helical conformer of β -glucan; TNF- α , tumor necrosis factor- α

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essential for host survival from infection, and is also required for the repair of tissue injury. These beneficial effects, however, are critically dependent on the magnitude of the immune response, because large amounts of inflammatory mediators can also cause collateral damage to normal cells.

Nitric oxide (NO) also contributes to microbicidal effect. It is synthesized from L-arginine by nitric oxide synthase (NOS) in numerous mammalian cells and tissues (Nathan and Xie, 1994). Reaction of NO and superoxide generate ONOO⁻, which induces apoptosis in some human tumor cells (Lin et al., 1995), and it is also directly cytotoxic by causing DNA damage and oxidative tissue damage (Ischiropoulos and al-Mehdi, 1995; Szabo et al., 1996).

Nuclear factor- κ B (NF- κ B), a transcription factor, is involved in regulating the transcription of many of the immunomodulatory mediators (Chen et al., 1999). NF- κ B exists within the cytoplasm in an inactive form associated with regulatory proteins, called inhibitors of κ B (I- κ B). When stimulated by various extracellular signals, including lipopolysaccharide (LPS), signal cascades lead to phosphorylation of I- κ B, which is then ubiquitinated, thereby releasing NF- κ B dimers from the cytoplasmic NF- κ B–I- κ B complex, and allowing them to translocate to the nucleus (Chen et al., 1995). Several researches revealed that β -glucans are able to activate NF- κ B, thus induce the expression of proinflammatory cytokines (Kougiaris et al., 2001; Young et al., 2001).

The biopotency equivalence of mycelia of *Ganoderma lucidum* and the fruiting body on innate immunity is controversial. The main goals of this research were to study the immunomodulatory activity and mechanism of dried mycelia of *Ganoderma lucidum* produced by submerged cultivation on the induction of innate immune response, including TNF- α , IL-6, IFN- γ and NO release as well as NF- κ B activation, in human blood cells or murine macrophage cell line.

2. Materials and methods

2.1. Materials

The commercialized spray dried *Ganoderma lucidum* (GL) mycelia is produced by Taiwan Sugar Co. (Taipei, Taiwan) by submerged cultivation. The polysaccharide content of GL is 3.72% and β -glucan content is 0.2% measured by aniline-blue binding assay (Young and Jacobs, 1998). Murine macrophages, RAW264.7, were purchased from Biosources Collection and Research Center (Hsinchu, Taiwan). Fetal bovine serum (FBS) was obtained from ICN Biomedicals (Irvine, CA, USA). Dulbecco's modified eagle's medium (DMEM) was from Invitrogen Life Technologies (Carlsbad, CA, USA). Non-essential amino acid, pyruvate, penicillin and streptomycin were obtained from Biochrom Beteiligungs GmbH&Co. (Berlin, Germany). LPS and Griess reagent were obtained from Fluka Chemika (Buchs, Switzerland). Hepes buffer and RPMI-1640 medium were obtained from Sigma (St. Louis, MO, USA). NE-PER Nuclear, Cytoplasmic Extraction Reagent, Cytokine ELISA Kit, Bradford Kit and LightShift Chemiluminescent EMSA Kit were purchased from Pierce Endogen (Rockford, IL, USA). Ficoll-

Paque Plus was purchased from Amersham Bioscience (Uppsala, Sweden).

2.2. Culture of human whole blood and cytokine measurement

Heparinized venous blood was obtained from four healthy volunteers and 1:10 diluted with cell culture medium (RPMI1640) supplemented with penicillin-streptomycin in 24-well plates. Cells were maintained in a humidified incubator at 37 °C in 5% CO₂. Diluted human whole blood was treated with saline, LPS (0.25 μ g/ml), PHA (10 μ g/ml) or GL (0.2–1.6 mg/ml) plus polymyxin B (10 μ g/ml) to eliminate the possible contamination by the endotoxin—a lipopolysaccharide from the Gram negative bacterial cell wall (LPS) of our samples (Cinco et al., 1996). TNF- α and IL-6 release in the supernatant was measured after 8 h incubation and IFN- γ production was measured after 3 days of incubation. Cytokines were measured using ELISA Kits.

2.3. Culture and measurement of TNF- α and nitrite release in RAW264.7 cells

RAW264.7 cells were cultured in DMEM with 10% fetal bovine serum, 2 mM glutamine, 1% non-essential amino acid, 1 mM pyruvate, 100 U/ml penicillin and 100 μ g/ml streptomycin. RAW264.7 cells were cultured with LPS (0.25 μ g/ml) or GL (0.4–1.0 mg/ml) plus polymyxin B (10 μ g/ml) in a humidified incubator at 37 °C in 5% CO₂. TNF- α in the supernatant was measured after 8 h treatment by ELISA Kit. Nitrite production, an indicator of NO synthesis, was determined by the Griess reaction after 24, 48 and 72 h treatment. The supernatant of RAW264.7 cell culture was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% aphythylenediamine in 5% phosphoric acid). The optical density at 550 nm (A_{550}) was measured and calculated against a sodium nitrite standard curve.

2.4. Electrophoretic mobility shift assay (EMSA)

RAW264.7 cells (1×10^6 cells/ml) were grown in six-well plates and stimulated with LPS (1 μ g/ml) or GL (1.6 mg/ml) plus polymyxin B (10 μ g/ml) at 37 °C in 5% CO₂ for 30–120 min. Nuclear extracted were prepared by NE-PER Nuclear and Cytoplasmic Extraction Reagent. EMSA experiment was performed using a LightShift Chemiluminescent EMSA Kit. Briefly, 20 μ g of nuclear protein was incubated with 20 fmol of 5'-biotinate double-stranded oligonucleotided probes containing a consensus binding-sequence for NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGGC-3') for 30 min at room temperature and resolved in an 8% non-denaturing polyacrylamide gel. The protein–DNA–biotin complexes were blotted onto a nylon membrane followed by UV cross-linking. The complexes were revealed with streptavidin-horseradish peroxidase conjugate and LightShift chemiluminescent substrate.

2.5. Statistical analysis

The results were analyzed by Student's unpaired *t*-test, and a *p* value of <0.05 was taken to be significant.

3. Results

3.1. Effect of GL on cytokine release

Since we were interested in investigating the influence of GL mycelia on the effector cells of the innate immune system, in addition to traditional macrophage cell line system, we chose the *in vitro* cytokine release from human whole blood as a convenient and simple surrogate approach to characterize changes in immune function of human. The level and persistence of TNF- α , IL-6 and IFN- γ plays an important role in determining its role in immunomodulation. Fig. 1 demonstrated that treatment of GL for 8 h dose-dependently induced moderate TNF- α and IL-6 release of human whole blood. When GL concentration was higher than 0.8 mg/ml, the TNF- α and IL-6 production reached plateau with 532.17 ± 110.71 and 365.93 ± 138.49 pg/ml, respectively. On the other hand, the levels of TNF- α and IL-6 from the positive control, LPS (0.25 μ g/ml)-stimulated whole blood, were much higher with 1472.83 ± 216.11 and 2221.45 ± 20.97 pg/ml, respectively. None of the treatment exerted significant cytotoxicity as determined by trypan blue exclusion assay (data not shown).

TNF- α is produced mainly by activated macrophages and T-cells, to specifically study the effect of GL on the activation of macrophages; we used murine macrophage cell line, RAW264.7 cells, as a model. Fig. 2 showed that RAW264.7 cells treated with GL (0.4–1.6 mg/ml) plus polymyxin B (10 μ g/ml) for 8 h evoked a concentration-dependent increase of TNF- α production and the maximal release was observed with 1.2 mg/ml GL. In contrast to the moderate level stimulated by GL (less than 3613.50 ± 42.43 pg/ml), TNF- α release from LPS (1 μ g/ml) activated macrophages was high (10821 ± 201.53 pg/ml).

IFN- γ is produced mainly by T-cells and natural killer cells, which are activated by antigens, mitogens, or alloantigens. IFN- γ increases macrophage and natural killer cell functions, as well as MHC class I and II cell surface antigen expression. To better understand the effect of GL on the induction of innate immunity, the IFN- γ release from human whole blood treated with GL (0.2–1.0 mg/ml) or PHA (10 μ g/ml) was measured. Table 1 demonstrated that GL (0.2–1.0 mg/ml) induced IFN- γ production in a dose-dependent manner with highest release of 43.13 ± 3.2 pg/ml; on the other hand, the T-cell mitogen, PHA (10 μ g/ml), stimulated human whole blood to release large quantity of IFN- γ (2762.44 ± 261 pg/ml).

3.2. Effect of GL on NO release

Nitric oxide (NO) has been associated with protection host against various parasitic, bacterial and viral infections (Tsai et al., 1997). Fig. 3 showed that RAW264.7 macrophages treated with GL (0.4–1.6 mg/ml) did not significantly stimulated nitrite

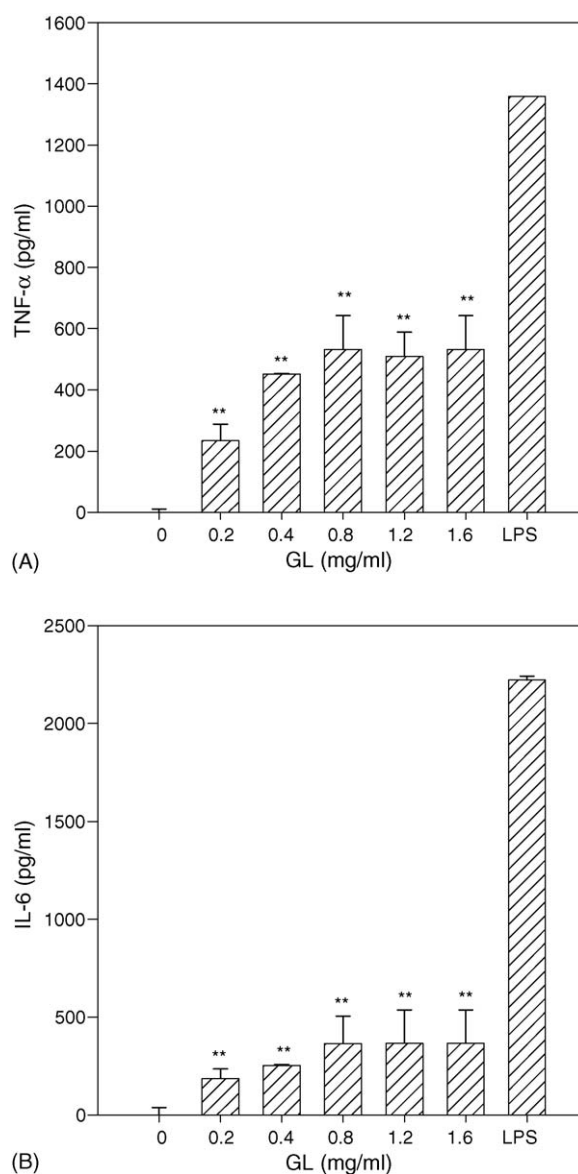


Fig. 1. *Ganoderma lucidum* mycelia induce the production of TNF- α and IL-6 in human whole blood. Human whole blood was stimulated with LPS (0.25 μ g/ml) or *Ganoderma lucidum* mycelia (0.2–1.6 mg/ml) plus polymyxin B (10 μ g/ml) for 8 h. Supernatants were collected for cytokine analysis by ELISA. (A) TNF- α release; (B) IL-6 release. Data are represented as the means \pm SEM of three independent experiments. **p* < 0.05 and ***p* < 0.01 represent significant differences compared to the control.

Table 1

IFN- γ release from the culture supernatants of human whole blood incubated with dried mycelium *Ganoderma lucidum* and polymyxin B (10 μ g/ml) for 72 h

Treatment	IFN- γ release (pg/ml) ^a
Control	Undetectable
<i>Ganoderma lucidum</i> (0.2 mg/ml)	7.33 \pm 1.21
<i>Ganoderma lucidum</i> (0.4 mg/ml)	25.01 \pm 2.10
<i>Ganoderma lucidum</i> (0.8 mg/ml)	35.01 \pm 1.25
<i>Ganoderma lucidum</i> (1.0 mg/ml)	43.13 \pm 3.2
PHA (10 μ g/ml)	2762.44 \pm 261

^a Values are means \pm SEM of three independent analyses.

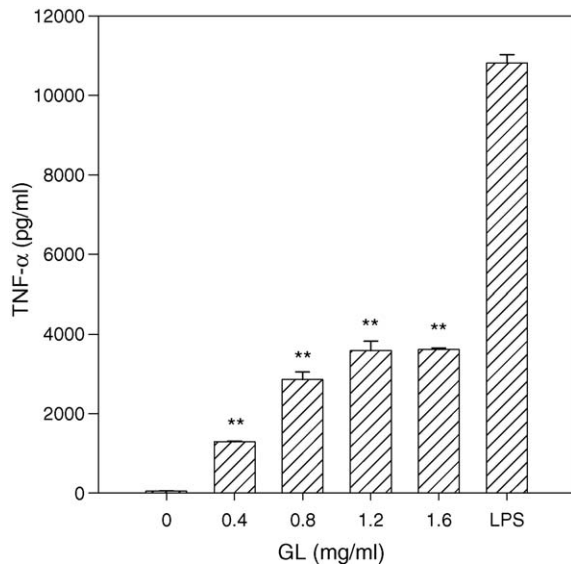


Fig. 2. *Ganoderma lucidum* mycelia induces the production of TNF- α in RAW264.7 cells. RAW264.7 cells were treated with LPS (1 μ g/ml) or *Ganoderma lucidum* mycelia (0.2–1.6 mg/ml) plus polymyxin B (10 μ g/ml) for 8 h. Supernatants were collected for TNF- α analysis by ELISA. Data are represented as the means \pm SEM of three independent experiments. * p < 0.05 and ** p < 0.01 represent significant differences compared to the control.

release as compared with control groups. In contrast, stimulation of macrophages with LPS (1 μ g/ml) induced an increase in nitrite release (p < 0.01). After 12 h of treatment, nitrite release increase from basal level 0.12 ± 0.08 to 25.17 ± 0.91 μ M. LPS-stimulated nitrite production reached plateau after 24 h and remained at high level for at least two more days.

3.3. Effect of GL on NF- κ B activation

NF- κ B is a converging point of various immune and inflammatory responses. To gain more insight into the mechanism of GL-mediated innate immune response, we analyzed the

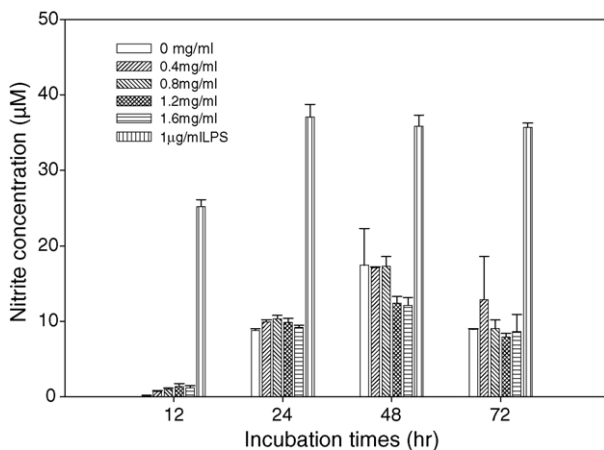


Fig. 3. Nitrite production in RAW264.7 cells. RAW264.7 cells were treated with LPS (1 μ g/ml) or *Ganoderma lucidum* mycelia (0.4–1.6 mg/ml) plus polymyxin B (10 μ g/ml) for 24, 48 and 72 h. Supernatants were collected for nitrite analysis as described in Section 2. Data are represented as the means \pm SEM of three independent experiments.

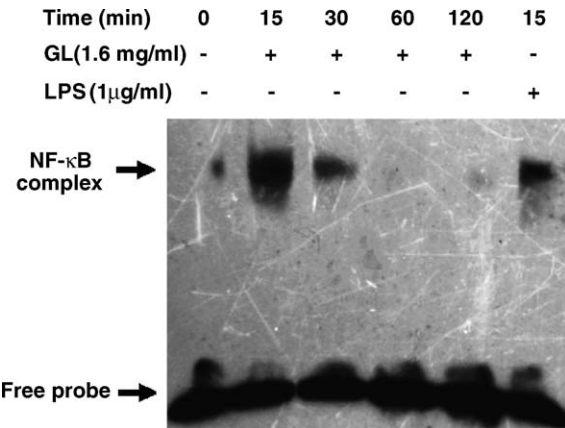


Fig. 4. Effects of *Ganoderma lucidum* mycelia on κ B DNA binding in RAW264.7 cells. RAW264.7 cells were cultured with LPS (1 μ g/ml) or *Ganoderma lucidum* mycelia (1.6 mg/ml) plus polymyxin B (10 μ g/ml) for various time. Nuclear extracts were prepared and analyzed for κ B DNA binding using the electrophoretic mobility shift assay (EMSA).

κ B-DNA-binding activity present in nuclear extracts of GL-stimulated macrophages. RAW264.7 cells were treated with GL (1.6 mg/ml) and the nuclear extracts were isolated at different time points (15, 30, 60 and 120 min) followed by electrophoretic mobility shift assay. Fig. 4 showed that GL induced-NF- κ B activation reached the maximum after 15 min treatment and inactivation occurred after 60 min. LPS (1 μ g/ml)-treated RAW264.7 was employed as a positive control to verify the shift band.

4. Discussion

In this study, we used commercial dried mycelia of *Ganoderma lucidum* as a sample to investigate its bioactivity and mechanism. We focused on the innate immunity augment activity of induction cytokine synthesis. We found that *Ganoderma lucidum* mycelia stimulated moderate levels of TNF- α , IL-6 and IFN- γ release in human whole blood. This induction is not due to endotoxin contamination since polymyxin B has been included in the experiments. It has been shown that the addition of polymyxin B is a useful technique to exclude the effects of endotoxin contamination (Duff and Atkins, 1982). The magnitudes of cytokine release are less than what LPS or PHA induces, suggesting the possible beneficial effect of cytokine induction by *Ganoderma lucidum* mycelia on innate immunity.

It has been demonstrated that crude water-extracted polysaccharides isolated from fresh fruiting bodies of *Ganoderma lucidum* potentiated the production of cytokines, including IL-1 β , IL-6, IFN- γ and TNF- α as well as nitric oxide (NO) and other mediators by human macrophages (Lee et al., 1995; Wang et al., 1997; Han et al., 1998). The crude *Ganoderma lucidum* water-extract also induced the expression of cytokines, including IL-10 and TNF- α , IL-1 β , IL-6 and IL-2 in human PBMC (Mao et al., 1999).

However, we found that *Ganoderma lucidum* mycelia can moderately stimulate cytokine production without potentiating NO release. It was found that the single helical conformer of β -glucan (SPG-OH), but not triple helical (SPG), enhanced NO

synthesis *in vitro*, especially in the presence of IFN- γ . In addition, the stimulation of IL-1, IL-6 and TNF- α production by SPG-OH were significantly higher than those by SPG (Ohno et al., 1996). Therefore, the ineffectiveness in inducing NO release by *Ganoderma lucidum* mycelia indicates that the compositions and structures of β -glucan in mycelia and fruiting body may be different, and this might result in enhancing innate immune response through different receptors or pathways.

Preliminary evidences showed that NF- κ B was activated in β -glucan activated human monocyte cell line U937 cells (Battle et al., 1998). In this study, we demonstrated that the dried mycelia of *Ganoderma lucidum* also induced NF- κ B activation in murine RAW264.7 macrophage cell line, indicating NF- κ B activation is one of the most important signal pathways. Proinflammatory cytokines (TNF- α , IL-1 β or IFN- γ) can bind to their respective receptors and induce iNOS expression via activation of NF- κ B (Xie et al., 1994; Pahan et al., 1998). However, we have found that the induction of NF- κ B does not lead to an increase in NO production. It has been found that the expression of the iNOS gene can be regulated at different levels (Nathan and Xie, 1994; Rao, 2000), therefore, *Ganoderma lucidum* mycelia may exert adverse effects on iNOS gene expression post-transcriptionally. Recently, it has been demonstrated that exposure of neutrophils to the polysaccharide from *Ganoderma lucidum* time dependency caused increases in protein kinase C (PKC), p38 mitogen-activated protein kinase (MAPK), Hck, and Lyn activities (Hsu et al., 2003; Lin et al., 2003). The involvement of MAPKs pathway by *Ganoderma lucidum* mycelia in macrophages is still under investigation.

Acknowledgements

We thank Dr. Cheng in TaiSugar Research Institute for supplying *Ganoderma lucidum* mycelia and Ms. L.L. Hsia and Y.M. Chen for their assistance in sample preparation.

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