Effects of *Ganoderma lucidum* Polysaccharide on CYP2E1, CYP1A2 and CYP3A Activities in BCG-Immune Hepatic Injury in Rats

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The purpose of the present study was to investigate the effect of *Ganoderma lucidum* polysaccharide (GLPS), a major active component in Chinese medicinal fungus, on cytochrome P450 metabolic activity in *Bacillus Calmette Guérin* (BCG)-induced immune hepatic injury in rats. The enzyme kinetics of the probes including chloroxazone (CYP2E1), phenacetin (CYP1A2) and nifedipine (CYP3A) were evaluated by HPLC. The results showed that BCG-pretreatment (125 mg/kg) significantly increased serum levels of alanine transaminase (ALT), nitrite and malondialdehyde (MDA), inhibited activities of superoxide dismutase (SOD) and decreased P450 total content in microsomes (p<0.05). Administration of GLPS (50 and 200 mg/kg) reversed above hepatic injury stimulated by BCG *in vivo*. Moreover, GLPS dose-dependently inhibited activities of CYP2E1, CYP1A2 and CYP3A in hepatic microsomes *in vitro*, suggesting that inhibition of GLPS on P450 oxidative metabolism might participate in the hepatoprotective mechanism, and also suggested that pharmacokinetics might be changed by drug–herb interaction.

**Key words** *Ganoderma lucidum* polysaccharide (GLPS); cytochrome P450; immune hepatic injury; *Bacillus Calmette Guérin* (BCG); HPLC; rat

*Ganoderma lucidum* (LEYSS, ex Fr., *G. lucidum*) Karst has been prescribed to improve health and longevity in the traditional Chinese medicine, and it was used for the treatment of neurasthenia, hypertension, hepatopathy and carcinoma for thousands of years. More than one hundred species of bioactive components have been isolated from *G. lucidum*, such as polysaccharides, triterpenoids and alkaloids, in which that *Ganoderma lucidum* polysaccharide (GLPS) is one of the major active components. Its multiple pharmacological effects, such as antitumor, antioxidation, immunomodulation and especially hepatoprotection against chemical or immune hepatic damage, have been demonstrated in many animal models *in vivo* and *in vitro*. However, the accurate protective mechanism of GLPS on the liver damage is still unknown.

Cytochrome P450 (P450) monooxygenase superfamily is the most important phase I metabolic enzyme system in liver. P450 is not only responsible for the oxidative metabolism of numerous exogenous compounds and endogenous hormones, but also plays a critical role involving in the activation of various chemical toxicant and procarcinogen. There are researches which have confirmed that the expressions and activities of some P450 isoenzymes may be down-regulated under the inflammatory or infecting condition, and then resulting in change of the metabolic capability of the enzymes. However, whether P450 down-regulation is a homeostatic mechanism or a pathophysiological phenomenon has not been elucidated. On the other hand, because *G. lucidum* is currently popularly used as self-medication in East Asia, thus, it is needed to be investigated that whether GLPS influences P450 metabolic activity.

*Bacillus Calmette Guérin* (BCG), a live attenuated vaccine, is clinically used as a preventing vaccine for tuberculosis or therapeutic regimens for bladder cancer. On the other hand, some cases of disseminated BCG infection have been proven to induce granulomatous hepatitis, sepsis and multiple organ failure in these patients. Several animal experiments have confirmed that BCG infection could induce the immune hepatic injury and production of inflammatory cytokines, active free radicals and nitric oxide (NO). Moreover, it was reported that microsomal P450 content and activity were suppressed by NO from inducible nitric oxide synthase (iNOS) during BCG infection in rodent liver. Therefore, the pharmacokinetics of P450 metabolized substrates may be changed by BCG in clinical patients, leading to enhancement of the therapeutic or adverse effects of drugs.

In our previous experiment, it has been observed that GLPS inhibits iNOS expression or NO production, and mitigates immune liver injury induced by BCG in mice *in vivo* and *in vitro*. The purpose of the present study was to investigate the effect of GLPS on the metabolic activities of three P450 isoenzymes including CYP2E1, CYP1A2 and CYP3A, which are abundantly expressed in liver, and further to evaluate whether GLPS modulating P450 activity involved in the hepatoprotective mechanism under the similar damaged condition with our previous study in rats.

**MATERIALS AND METHODS**

**Chemicals and Reagents** Nifedipine, oxidized nifedipine, 6-hydroxychloroxazone, β-nicotinamide-adenine dinucleotide phosphate, reduced form (β-NADPH), and 8-chlorotheophylline were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), and phenacetin from Fluka Chemie GmbH (Buchs, Switzerland). *Mycobacterium bovis* BCG vaccine, acetaminophen and chloramphenicol were ordered from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Chloroxazone was kindly provided by Professor Xiunei Zhang, School of Medicine, Shandong University (Shandong, China). All other chemicals and solvents used were of analytical grade.

**Preparation of GLPS** GLPS was provided by Fuzhou Institute of Green Valley Bio-Pharm Technology (Fuzhou, China). The preparation, purification and identification of GLPS were performed as previously described. Briefly, GLPS was extracted by hot water from the *G. lucidum* fruit...
ing body (No. of strain Ga0801), followed by ethanol precipitation, dialysis, and protein depletion using the Sevag method. The total yield of GLPS was 0.82% (w/w) in terms of the 

*G. lucidum* fruiting body. The purity and molecular weight distribution of GLPS were determined by gel permeation chromatography (GPC) and HPLC. Monosaccharide composition was determined by gas chromatography (GC). The structure of the glycopeptides was detected by IR, 1H- and 13C-NMR. The GLPS was a peptide-bound polysaccharide consisting of approximately 93.61% polysaccharide and 6.49% peptides. GLPS had a molecular weight of 584900, and the peptide part was 1H- and 13C-NMR.

The activities of CYP2E1, CYP1A2 and CYP3A isozymes were assayed using commercial analysis kits obtained from Beijing Bei Hua (SOD) were detected using commercial kits. The activities of CYP2E1, CYP1A2 and CYP3A isozymes were assayed using commercial analysis kits obtained from Beijing Bei Hua. The activities of CYP2E1, CYP1A2 and CYP3A isozymes were assayed using commercial analysis kits obtained from Beijing Bei Hua.

**Animals Treatment and Liver Damage Induction**

Male Sprague-Dawley rats (body weight 250±30 g) were purchased from the Department of Experimental Animals, Beijing University (Beijing, China). The animal studies were approved by the Animal Ethics Committee of Beijing University, and carried out in accordance with the requirements of China national legislation.

Thirty rats were randomly divided into five groups: control, BCG, BCG plus GLPS (50 or 200 mg/kg) and GLPS (200 mg/kg) group. Immune hepatic injury was induced by intravenous injection of BCG (125 mg/kg) for two weeks in rats as described in our previous report.7 The choice of doses and time period of GLPS was also based on our previous experiment and other reports.7,16,17 After BCG-pretreatment for 7 d, different doses of GLPS were intragastric administered once a day and continued within the succedent one week. At the end of two weeks of immune stimulation, rats were sacrificed by decapitation; blood sample (1 ml) was collected for serum biochemical analysis. Liver and spleen were removed rapidly and weighted. Livers were stored at −70 °C until use. Protein concentrations were estimated using the Bradford assay.19 Total P450 content was assessed using commercial kits obtained from Beijing Bei Hua. The activities of CYP2E1, CYP1A2 and CYP3A isozymes were assayed using different probe drugs by HPLC. Liver microsomes were obtained from another twelve rats in absence or presence of BCG pretreated group as above described methods. The HPLC system consisted of a Waters 510 pump with 7725i injector, Waters 490 UV-detector and a reversed phase HPLC column (Alltima C18 analytical column, 150×4.6 mm, 5 μm, Alltech, U.S.A.). The flow-rates of mobile phases were all 1.0 ml/min.

**Chloroxazone 6-Hydroxylation**

CYP2E1 activity was determined via chloroxazone 6-hydroxylation metabolism in liver microsomes in vitro.25 The incubation mixture in 0.5 ml total volume contained 0.6 mg/ml microsomal protein, 0.1 M potassium phosphate buffer (pH 7.4), and 500 μM chloroxazone (10 μl of 25 mM stock solution) and was preincubated for 5 min at 37 °C. The reactions were initiated by the addition of 50 μl β-NADPH (final 0.8 mM) and continued for 20 min, and then terminated by 3 ml ice-cold ethyl acetate. Then 40 μl chloramphenicol (64 μg/ml) was added as the internal standard. The extracted residue was dissolved in 500 μl mobile phase (acetonitrile and 0.8% acetic acid 35 : 65, v/v) and 20 μl was injected into the HPLC system with UV 287 nm for analysis.

**Phenacetin O-Deethylation**

Hepatic CYP1A2 activity was assayed via phenacetin O-deethylation.23 In 1 ml total reacting volume, 0.2 mg/ml microsomal protein and 50 μM phenacetin (100 μl, 0.5 mm stock solution) were preincubated for 5 min at 37 °C. The final concentration of β-NADPH was 0.5 mM. Then 40 μl 8-chlorotheophylline (160 μg/ml, internal standard) was used. Other conditions of incubation and extraction were similar with described above. The extracted sample was dissolved in 200 μl mobile phase (20 mM acetic sodium buffer and methanol 2 : 1, v/v) and 30 μl was analyzed by HPLC with UV 254 nm.

**Nifedipine Oxidation**

CYP3A activity was evaluated by nifedipine oxidation as a specific probe reaction.20 The total volume of incubation was 0.5 ml, which containing 0.4 mg/ml microsomal protein, 10 μl nifedipine (final 200 μM) and 0.8 mM β-NADPH (50 μl of stock solution), incubating for 10 min. The reaction was stopped by 100 μl cold 1 mM sodium carbonate (Na2CO3) with 2 mM sodium chloride (NaCl, pH 10.5). Phenacetin 50 μl (2 μg/ml) was used as the internal standard. The extracted residue was redisolved in 200 μl mobile phase (methanol and water, 64 : 36, v/v) and 20 μl was measured by HPLC with UV at 254 nm.

**Statistical Analysis**

All data were represented as a mean ± standard deviation (S.D.) obtained from six independent experiments. Statistical analysis were determined by One-way analysis of variance (ANOVA), followed by Dunnett t test (2-sided). p values below 0.05 were considered as statistically significant.

**RESULTS**

**Effect of GLPS on BCG-Induced Hepatic Injury in Rats in Vivo**

Compared with control group, BCG-pretreatment significantly increased the ratio of organ weight relative to body weight of both liver and spleen (Figs. 1A, B, p<0.01). Similarly, the serum hepatic enzyme levels of both ALT (Fig. 1C, p<0.05) and AST (Fig. 1D, p<0.01) were significantly enhanced by BCG-stimuli in rats. Administration of GLPS decreased above ALT and AST levels in the range...
of 50 to 200 mg/kg in BCG-stimulated rats. Inhibitory effects of GLPS on the hepato- and splenomegaly induced by BCG were shown the markedly trend, in despite of significant differences were not observed. Moreover, application of GLPS markedly improved the pathological changes induced by BCG stimulation, such as infiltration within liver lobules by inflammatory cells, hemorrhage and granulomas formation in liver parenchyma, similarly to our previous observation in mice.7) On the other hand, no marked changes of liver enzymes levels were observed in GLPS alone treated (200 mg/kg) group in rats, excluding the potential influence of GLPS on liver in the control rats.

Effects of GLPS on NO Production, Lipid Peroxidation and SOD Activity in BCG-Induced Hepatic Injury in Rats in Vivo As shown in Fig. 2, BCG-pretreatment significantly increased the levels of nitrite in serum and MDA in liver microsomes, moreover, reduced microsomal SOD activity in rats in vivo. Whereas, administration of GLPS decreased the serum nitrite levels and microsomal MDA content, enhanced SOD activity in the range of 50 and 200 mg/kg under the presence of BCG stimulating condition in rats.

Effects of GLPS on P450 Total Contents in BCG-Induced Hepatic Injury in Rats in Vivo Although there was no marked difference of microsomal protein concentration among control, BCG-stimulated and GLPS-treated groups in rats (Fig. 3A), BCG-pretreatment significantly decreased P450 total contents in rat microsomes in vivo (Fig. 3B, p<0.01). GLPS (50, 200 mg/kg) partly restored the P450 contents with a dose-dependent manner, but no significant changes were observed.

Effect of GLPS on CYP2E1 Activity in BCG-Stimulated Hepatic Microsomes in Vitro As shown in Fig. 4A, the addition of GLPS (50, 100, 400, 800 μg/ml) decreased the CYP2E1 catalyzed activity with the concentration-dependent manner in the range of 400 and 800 μg/ml (p<0.05). The estimated IC50 of GLPS was 743 μg/ml.

Furthermore, pretreatment with BCG (125 mg/kg, i.v. 2 weeks) significantly inhibited the metabolic rate of chlorzoxazone 6-hydroxylation in hepatic microsomes (p<0.01). Under the BCG-pretreatment condition, the addition of GLPS induced the decreasing trend of CYP2E1 catalyzed activity, in despite of significantly change was not observed within the tested range in vitro.

Effect of GLPS on CYP1A2 Activity in BCG-Stimulated Hepatic Microsomes in Vitro As shown in Fig. 4B, after co-incubation with the different concentrations of GLPS (10, 50, 100, 400, 800 μg/ml), the CYP1A2 activity was significantly inhibited in the range of 400 and 800 μg/ml in hepatic microsomes (p<0.01). The IC50 value of GLPS...
was estimated as 393 μg/ml.

Moreover, pretreatment with BCG reduced the metabolic rate of phenacetin O-deethylation to 0.223±0.061 nmol/min/mg protein. Under the BCG-pretreatment condition, the addition of GLPS further decreased the CYP1A2 catalyzed activity within the range of 400 to 800 μg/ml in vitro (p<0.01). The IC50 value of GLPS was estimated as 424 μg/ml.

**Effect of GLPS on CYP3A Activity in BCG-Stimulated Hepatic Microsomes in Vitro** In the absence of BCG pretreatment group, GLPS dose-dependently suppressed the hepatic microsomal CYP3A metabolic activity within the tested range (6.25, 25, 100, 400, 800 μg/ml), and the significantly difference was observed in 800 μg/ml group (p<0.01). The IC50 value of GLPS was 783 μg/ml (Fig. 4C).

Similarly, pretreatment with BCG significantly decreased the metabolic rate of nifedipine oxidation in rat hepatic microsomes (p<0.01). Under the BCG-pretreatment condition, the addition of GLPS induced the decreasing trend of CYP3A catalyzed activity, in spite of significantly change was not observed within the range of 6.25 to 800 μg/ml in vitro. It was speculated that the level of CYP3A activity was very lower in BCG-pretimulated microsomes in vitro, so that the inhibitory degrees by GLPS might be less than that observed in control microsomes.

**DISCUSSION**

BCG infection has been proven to be a kind of cell-mediated immune response. Pretreatment with BCG activates macrophages and T lymphocytes within hepatic granulomas to release several kinds of proinflammatory cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). These cytokines further stimulate liver parenchymal and nonparenchymal cells to produce several important inflammatory mediators, such as reactive oxygen species (ROS) and reactive nitrogen intermediates including NO, which could cause oxidative damage to cellular components. The present results indicated that BCG-pretreatment significantly increased the serum levels of ALT and nitrite, enhanced the MDA content and inhibited the activity of SOD in microsomes. Administration of GLPS decreased the levels of above biomarkers, such as lipid peroxidation or oxidative stress in BCG-induced hepatic injury in rats. This result was consistent with the hepatoprotective effect of GLPS observed in our previous experiments, for example, which mitigated infiltration of inflammatory cells, hemorrhage and granulomas formation in liver sections in BCG-stimulated mice. Moreover, other researches also confirmed that NO production from iNOS was involved in hepatotoxicity induced by BCG infection in mice. Therefore, the present result suggested that hepatoprotective mechanism of GLPS for BCG-induced immune liver injury might be due to inhibition of oxidative stress in liver.

The present results further demonstrated that BCG-pretreatment significantly decreased hepatic P450 total content, and inhibited activities of CYP2E1, CYP1A2 and CYP3A in liver microsomes, conjecturing that this P450 down-regulation might be a homeostatic mechanism in order to protect the cell from the deleterious effects of these oxidizing species after BCG-immune stimulation. The present results revealed that GLPS dose-dependently inhibited CYP3A, CYP2E1 and CYP1A2 catalyzed activities in hepatic microsomes, supporting the opinion that inactivation of P450 isoenzymes metabolic ability in rats. Several researches revealed that rat CYP3A enzymes could form NO from N-hydroxarginine in hepatocytes, aggravates lipopolysaccharide (LPS) and cytokines-induced liver injury by NO-dependent mechanism. In contrast, the administration of CYP3A inhibitors decreased LPS-stimulated production of NO and prevented hepatic damage. Furthermore, it has been observed that toxicity of carbon tetrachloride (CCl4) is dependent on CYP2E1 mediated formation of ROS and toxic metabolites, in consequence causing membrane lipid peroxidation, mitochondrial DNA oxidative damage and cell apoptosis. CYP1A2 is another phase I metabolic enzyme specifically expressed in the liver. It can be induced by some polycyclic aromatic hydrocarbons (PAHs), and takes a great part in oxidative metabolic activation of environmental toxicants and procarcinogens. Thus, the present results suggested that GLPS inhibited hepatic oxidative damage possibly due to inhibited above P450 isoenzymes metabolic ability in rats.
Moreover, the classical P450 inhibitor, such as metyrapone, may be a better choice for identifying the effects of P450 isoenzymes, and it will be applied in our further research.

On the other hand, an unexpected consequence was observed that the addition of GLPS partly restored the P450 contents decreased by BCG-stimulating, in spite of significant changes were not observed in the present study. Similarly, it was reported that loss of hepatic P450 activity resulted in increase of P450 protein content, by conditional deletion of hepatic cytochrome P450 reductase (CPR-null mice). These results suggested that there might be the existence of a negative feedback pathway regulating P450 expression, and the exact mechanism remains to be determined in the future. Furthermore, due to being a heme containing protein, P450 content might be affected by alteration of heme synthesis and degradation. It needs to be evaluated whether heme metabolism influences P450 activity in BCG-immune hepatic injury in the further research.

In conclusion, the present results demonstrated that GLPS alleviated hepatic injury stimulated by BCG in vivo, and inhibited metabolic activities of CYP1A2, CYP2E1 and CYP3A in rat hepatic microsomes in vitro. These results suggested that the inhibitory effects of GLPS on P450 oxidative metabolism might participate in the hepatoprotective mechanism.

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