Bilobol inhibits the lipopolysaccharide-induced expression and distribution of RhoA in HepG2 human hepatocellular carcinoma cells

JIN XU[1], YUEYING LI[1], XIAOMING YANG[2], YALI LIU[2], YONGCHANG CHEN[1] and MIN CHEN[1]

[1]Department of Anatomy and Physiology, School of Medicine; [2]Department of Food Science and Engineering,
School of Food and Biological Engineering, Jiangsu University, Zhenjiang, Jiangsu 212013, P.R. China

Abstract. Recent studies have revealed the localization of RhoA protein in the cell nucleus, in addition to its distribution in the cytosol and cell membrane. The results of previous studies by our group indicated that nuclear RhoA expression is increased, or RhoA is transported into the nucleus, when cells become cancerous or damaged. Furthermore, application of the anticancer agent Taxol appeared to reduce nuclear RhoA localization, indicating an association between the nuclear translocation of RhoA and tumor progression. Bilobol is a traditional Chinese medicine ingredient, however, its anticancer effect has remained unclear. The present study aimed to demonstrate the anticarcinogenic action of bilobol against hepatocellular carcinoma, in order to lay the foundations for subsequent research into the mechanisms underlying its anticancer effects. In the present study, HepG2 cells were treated with lipopolysaccharide (LPS), to induce inflammation, and/or bilobol. By performing an ELISA, it was observed that bilobol was able to suppress the inflammation induced by LPS. In addition, immunofluorescence and western blot analyses indicated that bilobol may reduce the expression of RhoA, suppress translocation of RhoA into the nucleus and inhibit the RhoA/Rho-associated protein kinase signaling pathway. In conclusion, the present study revealed the potential anticancer effects of bilobol.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortalities worldwide. Approximately 80% of HCCs develop in fibrotic or cirrhotic livers (1). Conventional treatment strategies include surgery, chemo-

therapy and radiotherapy. For the majority of patients with advanced cancer, chemotherapy must be administered following surgery. However, the prognoses of HCC patients, particularly those with high stage or chemoresistant tumors, are still unsatisfactory (2), and the range for radiation therapy is relatively narrow. Other optional treatments include immunotherapy and traditional Chinese medicine. Therefore, the current study investigated the role and mechanisms of bilobol, a traditional Chinese medicine ingredient, in order to understand its antitumor effects and to assess its potential as an antitumor agent or adjuvant agent.

RhoA is a small G protein, inactive when bound to guanosine diphosphate (GDP) and activated following guanosine triphosphate (GTP) binding. GDP/GTP exchange or GTPase reactions catalyze the conversion between the two forms (3). Rho, a small GTPase, is associated with various cellular functions, including cell adhesion, cell motility and migration, growth control, cell contraction and cytokinesis. Previous studies by our group (4,5) and others (6,7) have indicated that RhoA is located in the nucleus, as well as in the cell membrane and the cytoplasm. In addition, previous investigations conducted by the present research group demonstrated that the nuclear localization of RhoA was affected by numerous factors, including inflammation, damage and certain pharmaceutical agents (5). Furthermore, these previous studies revealed increased nuclear translocation of the RhoA protein when the cell in question is damaged or cancerous.

Bacterial lipopolysaccharide (LPS) is localized to the outer membrane of Gram-negative bacteria, and is capable of activating multiple mammalian cell types and intracellular signaling pathways (8). LPS is a major inflammatory molecule, which induces the production of pro-inflammatory toxins and cytokines, including inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-α and interleukin-1 (IL-1), IL-6 and IL-8, in various cell types (9). These pro-inflammatory cytokines and bacterial endotoxins induce activation of the Rho GTPase signaling pathway, which mediates pro-inflammatory responses and aggressive carcinogenesis (10,11).

Ginkgo biloba L. is a tree native to China, which has been cultivated for over a millennium. In Asia, the *Ginkgo biloba*

Correspondence to: Ms. Yueying Li, Department of Anatomy and Physiology, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013, P.R. China
E-mail: yyli@ujs.edu.cn

(Gb) tree has been used medicinally for centuries. Similarly, Gb leaf extracts are commonly used for a variety of traditional folk remedies in the West (12). Currently, Gb extracts are one of the most commonly administered phytomedicines world-wide, applied for the treatment of neuropsychiatric disorders, asthma, tinnitus, vertigo and cognitive issues (13,14).

The application of ginkgolic acids as anticancer agents has previously been reported (15,16). However, ginkgolic acids are unstable, with bilobol/ginkgo phenol forming easily following heat, acid or base catalysis (17). The cyto-toxic evaluation of bilobol as an antitumor agent is in the preliminary stages. Furthermore, an in-depth investigation into the mechanism and effects of bilobol on its targets has commenced (18-20). In consideration of previous studies regarding RhoA by our group, the present study aimed to conduct a broader investigation of the anticancer efficacy of bilobol by analyzing its effect on the distribution of RhoA in HepG2 human hepatocellular carcinoma cells.

Materials and methods

Cell line. The HepG2 human hepatocellular carcinoma cell line was obtained from the Institution of Cell Biology of the Chinese Academy of Sciences (Shanghai, China).

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Trypsin-EDTA solution were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fluorescein isothiocyanate (FITC), tetarhoda-mine isothiocyanate (TRITC) and horseradish peroxidase (HRP)-conjugated secondary antibodies [goat anti-mouse polyclonal IgG (cat no. 115-005-209) and rabbit anti-goat polyclonal IgG (cat no. 305-005-003)] were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Furthermore, electrochemiluminescence (ECL) reagents were purchased from GE Healthcare Life Sciences (Chalfont, UK). A Nuclear and Cytoplasmic Extract kit (cat no. KC-435) and antibodies against GAPDH were purchased from Kangchen Biotech, Inc. (Hangzhou, Zhejiang, China). Mouse monoclonal immunoglobulin G (IgG)₁ anti-RhoA (cat no. sc-418) and goat polyclonal IgG anti-Rho-associated protein kinase 2 (ROCK2; cat no. sc-1851) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). In addition, the ELISA kit was obtained from R&D Systems, Inc. (Minneapolis, MN, USA), LPS was from Sigma-Aldrich (St. Louis, MO, USA) and bilobol (high-performance liquid chromatography purity, >96.5%) was obtained from the Laboratory of Food and Biological Engineering School, Jiangsu University (Zhenjiang, Jiangsu, China).

Cell culture. HepG2 cells were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every two days and the cells were maintained at subconfluence.

Bilobol preparation and cell treatment. Bilobol was dissolved in 0.1% dimethyl sulfoxide (Bio-Link, York, UK) to obtain a concentration of 15 mg/ml. A total of 2.5x10⁶ HepG2 cells were seeded in 6 well plates for use in the Western
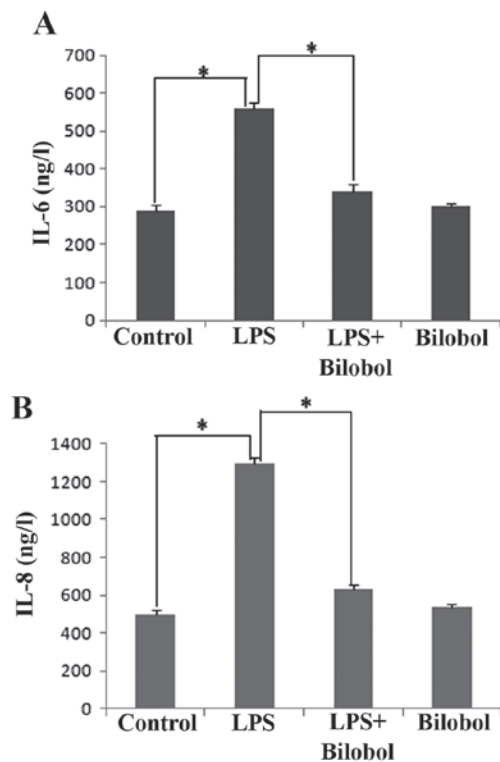

Figure 1. Bilobol rescues the LPS-induced increase in IL-6 and IL-8 expression. Expression of IL-6 and IL-8 induced by LPS or/and bilobol treatment in the HepG2 human hepatocellular carcinoma cell line. HepG2 cells were treated with or without 1 µg/ml LPS and 15 µg/ml bilobol for 12 h. Concentration of (A) IL-6 and (B) IL-8 in the supernatants, determined by ELISA. Data are presented as the mean ± standard error of the mean (n=6). *P<0.05. LPS, lipopolysaccharide; IL, interleukin.

blot analysis and IL-8/IL-6 expression assays and a total of 5x10⁵ HepG2 cells were seeded in 24 well culture plates for the immunofluorescence assays, and incubated in a humidified atmosphere with 5% CO₂ at 37°C. When the cells had reached ~80% confluence, DMEM medium with free serum was added to the wells. Next, 1 µM bilobol was added to the 6 and 24-well plates and the cells were cultured for 12 h. Untreated cells were used as the blank control.

Preparation of cytoplasmic and nuclear protein samples. The cytoplasmic and nuclear proteins were extracted from the cultured HepG2 cells according to protocol outlined in the Nuclear and Cytoplasmic Extract kit (Zhejiang Kangchen Biotech Co., Ltd., (Hangzhou, China) manufacturer's instructions. Briefly, the cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100; pH 7.4), collected in an Eppendorf tube (Genetimes Technology, Inc., Shanghai, China), centrifuged at 3,000 x g for 15 sec and suspended in 300 µl Buffer A. Phenylmethylsulfonyl fluoride (1 µl; Shanghai Bogoo Biotechnology Co. Ltd., Shanghai, China), a phosphatase inhibitor (aprotinin; Beijing Leagene Biotech Co., Ltd., Beijing, China), a protease inhibitor mixture, containing leupeptin (Beijing Leagene Biotech Co., Ltd.) and 12 µl NP-40 solution were added and the cells were placed on ice for 10 min, then vortexed for 10 sec. Next, the cells were placed on ice for 10 min and centrifuged at 12,000 x g for 15 sec. The supernatant, which contained
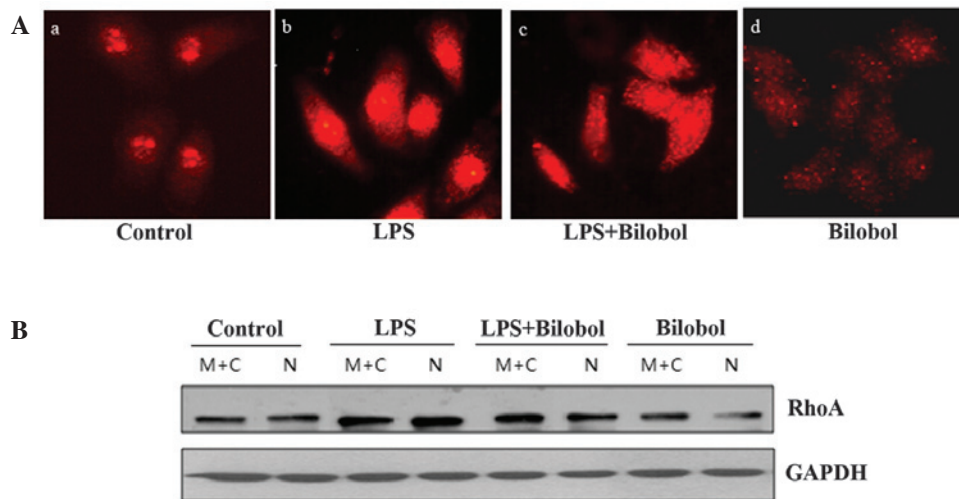
Figure 2. Bilobol inhibits the expression and nuclear localization of RhoA. Expression and distribution of RhoA induced by LPS or/and bilobol treatment in the HepG2 human hepatocellular carcinoma cell line. (A) RhoA was immunofluorescently stained with a specific mouse antibody (Aa) prior to treatment, or following the administration of (Ab) 1 μg/ml LPS, (Ac) LPS plus 15 μg/ml bilobol or (Ad) 15 μg/ml bilobol alone for 12 h (magnification, x400). (B) Three groups of HepG2 cells were lysed, and the M+C and N fractions were obtained. The expression of RhoA and GAPDH in each fraction was detected by western blotting, and GAPDH expression served as the loading control. LPS, lipopolysaccharide; M+C, membrane and cytosol; N, nucleus.
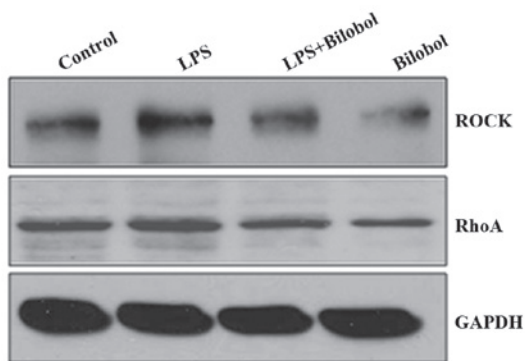

Figure 3. Bilobol inhibits the LPS-induced increased in ROCK2 expression. Protein expression levels of ROCK2 and RhoA induced by LPS or/and bilobol in the HepG2 human hepatocellular carcinoma cell line. Whole cell extracts were immunoprecipitated and protein expression was detected using ROCK2 and RhoA antibodies. GAPDH expression served as the loading control. LPS, lipopolysaccharide; ROCK2, Rho-associated protein kinase 2.

the cytoplasmic protein, was removed and transferred to an Eppendorf tube. The cells were then resuspended in 30 μl Buffer B containing protease inhibitors and placed on ice for 30 min. The cells were then centrifuged at 12,000 x g for 15 sec at 4°C and the supernatant was removed. This supernatant contained the nuclear protein. Equal volumes of 2X SDS loading buffer were added to the cytoplasmic and nuclear proteins, respectively. The proteins were then incubated in a Dry Bath Incubator (BG-themoRT; Bay Gene, Inc., Burlingame, CA, USA) at 100°C for 5 min.

Immunofluorescence microscopy. HepG2 cells cultured on cover slips were fixed with freshly prepared paraformaldehyde (40 g/l in phosphate-buffered saline; Nantong Jiangtian Chemical Co., Ltd., Nantong, Jiangsu, China) for 30 min. Following penetration with 30 ml/l Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and blocking with 30 g/l bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), the cells were incubated with primary antibodies (dilution, 1:200) at 4°C overnight (o/n). Subsequently, the cells were incubated with TRITC-conjugated polyclonal goat anti-mouse IgG secondary antibodies (dilution, 1:1,000) for 1 h at room temperature (RT). The cells were washed three times following each incubation. The cellular distribution of the target protein was analyzed under a fluorescence microscope (IX51; Olympus Corporation, Tokyo, Japan).

Western blotting. The sample proteins were run on 10.0 or 12% SDS polyacrylamide gels. Subsequently, the proteins were transferred onto polyvinyl difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PVDF membranes were initially blocked with 5% milk in Tris-buffered saline and Tween 20 (80 g/l NaCl, 2 g/l KCl, 30 g/l Tris and 0.1% Tween-20; pH 7.4) for 1 h at RT and then incubated with the primary antibodies (dilution, 1:1,000) at 4°C o/n. Following subsequent incubation of the membranes with HRP-conjugated secondary antibodies (dilution, 1:10,000) for 1 h at RT, ECL reagents were applied, according to the manufacturer' instructions, to reveal the positive bands on the membrane. The bands were detected using a Typhoon 9400 imager (GE Healthcare Life Sciences, Piscataway, NJ, USA).

Determination of IL-8 and IL-6 expression. HepG2 cells were treated with or without LPS (1 μg/ml) for 12 h. The supernatants were then harvested and measured for IL-8 and IL-6 production using an ELISA kit, according to the manufacturer's instructions.

Statistical analysis. All data are expressed as the mean ± standard error of the mean (n=6). The independent samples t-test was used to compare the expression of IL-6 and IL-8. The results were analyzed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Bilobol suppresses LPS-induced inflammation. The present study investigated whether LPS was able to mediate the transcriptional activation of the pro-inflammatory genes IL-6 and IL-8, and whether bilobol was able to suppress this LPS-induced inflammatory reaction. HepG2 human hepatocellular carcinoma cells were treated with 1 μg/ml LPS and/or 15 μg/ml bilobol for 12 h, and the concentration of IL-6 and IL-8 in the supernatants was measured by ELISA. The results indicated that LPS significantly increased the transcriptional activation of IL-6 (Fig. 1A) and IL-8 (Fig. 1B; P<0.05). Furthermore, bilobol was identified to significantly suppress the LPS-induced release of IL-6 (Fig. 1A) and IL-8 (Fig. 1B; P<0.05). Thus, bilobol exhibited an anti-inflammatory effect.

Bilobol inhibits the expression and nuclear translocation of RhoA. An immunofluorescence assay was performed, in order to determine the level of RhoA expression in the nucleus using an antibody against RhoA. Comparison with control cells revealed that LPS activated RhoA and triggered its translocation to the nucleus in HepG2 human hepatocellular carcinoma cells (Fig. 2Aa and Ab). Furthermore, treatment with bilobol appeared to suppress this effect (Fig. 2Ac and Ad). Thus, the expression of RhoA protein and its localization within the nucleus was increased when the cells were treated with LPS. Bilobol had the opposite effect; it was able to inhibit the two aforementioned phenomena. Subsequent to the immunofluorescent analysis, nuclear extracts were prepared and analyzed by western blotting, using antibodies against RhoA. The western blot analysis results were in agreement with the immunofluorescence data (Fig. 2B), indicating that bilobol inhibits the expression and nuclear translocation of RhoA.

Bilobol inhibits the expression of ROCK2. Western blotting was used to determine the expression of ROCK2 and RhoA, when HepG2 cells were treated with or without 1 μg/ml LPS and/or 15 μg/ml bilobol for 12 h (Fig. 3). The expression of ROCK2 was increased following LPS stimulation, however, treatment with bilobol was able to suppress its enhanced expression. ROCK2 appeared to follow a similar expression pattern to that of RhoA following treatment with LPS and/or bilobol.

Discussion

RhoA has a molecular mass of 21 kDa and is the most extensively studied member of the Rho GTPase family, which is part of the Ras superfamily of small G proteins. RhoA has been reported to regulate various biological activities, for example gene transcription (21) and tumor progression (22). Furthermore, RhoA activation is typically associated with invasive growth and metastasis, and therefore functions as an oncogene. RhoA is associated with the regulation of numerous biological processes, including stress fiber formation, membrane transport, gene transcription, focal adhesion and tumor progression (23,24).

Ginkgo biloba L. is the last surviving member of one of the oldest living seed plant groups, Gymnospermae, and is of medicinal, spiritual and horticultural importance worldwide. Gb extracts (EGb) are one of the most commonly administered therapeutic agents, and are often prescribed in Europe for use as nootropic agents in old age and dementia (25).

Investigation into the antitumor efficacy of the EGb alkylphenol, ginkgolic acid, has focused on ginkgo acid, specifically. Park *et al* (16) evaluated the anticancer effects of EGb in estrogen-independent breast cancer. It has been proposed that the chemopreventive effects exhibited by EGb in estrogen receptor-independent breast cancer occur via antiproliferative and apoptosis-inducing activities. Previously, Liu and Zeng (19) identified that HepG2 cells were more sensitive to the cytotoxicity of ginkgolic acid than primary rat hepatocytes. For example, ginkgolic acid appeared to significantly inhibit growth, halt G0/G1 phase progression and decrease B cell lymphoma-2 (Bcl-2)/Bcl-2-associated X protein expression in HepG2 cells (20), as well as induce the apoptosis of pituitary gland tumor cells by increasing the sensitivity of the cells to radiation (26).

However, alkylphenol acid obtained from EGb is unstable, rapidly forming bilobol/ginkgo phenol under heat, acid or base catalysis. Bilobol is considered to exhibit stronger anticancer properties.

In the present study, LPS appeared to stimulate the nuclear translocation of RhoA protein, while bilobol treatment blocked the subcellular distribution of RhoA under LPS-induced inflammatory conditions. Therefore, in consideration of the results of previous studies by our group, it was proposed that bilobol may exhibit anti-inflammatory and anticancer effects on HepG2 cells. Similarly, standardized EGb 761 and bilobalide treatment have been demonstrated to inhibit inflammation in rats; however, the mechanism of action has remained elusive (27,28). Furthermore, only a small number of studies have been conducted investigating the anti-inflammatory and anticancer effects of bilobol.

The best-known effector of RhoA is ROCK. ROCK appears to be crucial in modulating the force and velocity of actomyosin crossbridging in smooth muscle and non-muscle cells by inhibiting myosin phosphatase-mediated dephosphorylation of the regulatory chain of myosin II (29). In the present study, RhoA/ROCK expression and activity were increased following treatment with LPS, and reduced following the application of bilobol. In addition, bilobol appeared to inhibit the RhoA/ROCK signaling pathway during the anti-inflammatory response and exhibited an anticancer effect. However, the mechanism involved requires further clarification.

In conclusion, the present study identified that the administration of bilobol blocked cancer progression in HepG2 cells. This is in agreement with the results of previous studies by our group, which indicated that the nuclear translocation of RhoA promoted cancer progression. To further assess the antitumor effect of bilobol and develop it into a candidate novel antitumor agent, the antitumor mechanism of bilobol requires additional investigation.

Acknowledgements

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