Young Yum pill inhibits inflammatory mediators and nuclear factor-kappa B signaling in lipopolysaccharide-stimulated RAW 264.7 macrophages

Yin Chengle, Muhammad Jahangir Hossen, Anfernee Kai-Wing Tse, Su Tao, Fu Xiuqiong, Li Ting, Guo Hui, Zhu Peili, Li Junkui, Chou Jiyao, Wang Yaping, Yu Zhiling

Abstract

OBJECTIVE: To investigate the effect of Young Yum pill (YYP) on inflammatory mediators in cultured RAW 264.7 cells and elucidate the nuclear factor-kappa B (NF-κB)-related mechanism behind the action.

METHODS: YYP was extracted with 95% ethanol lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages were used to evaluate the effect of YYP on inflammatory mediators. Production of nitric oxide (NO) and prostaglandin E\(_2\) (PGE\(_2\)) were measured by Griess test and enzyme-linked immuno-
tion of inflammatory responses in the body will do harm to the host, resulting in the occurrence of chronic inflammation and even carcinogenesis. 1, 4 Macrophages, one type of immune effector cells, play a pivotal defensive role in the host against invading pathogens, such as bacteria, by engulfing them directly or killing them indirectly through various cytokines. 2 Lipopolysaccharide (LPS) is the major component derived from the outer membrane of Gram-negative bacteria that can initiate toll-like receptor (TLR)-4 signaling in macrophages, which may lead to the up-regulation of inflammation-related molecules such as inducible nitric oxide (NO) synthase (iNOS), cyclooxygenase-2 (COX-2) and inflammatory mediators such as NO, prostaglandin E2 (PGE2) and tumour necrosis factor-α (TNF-α). 2 These inflammatory mediators can further cause injury to the host. Therefore, suppression of the NF-κB pathway would be likely to alleviate damages caused by uncontrolled inflammation.

Young Yum pill (YYP), a proprietary drug, was developed based on a prescription of a Traditional Chinese Medicine (TCM) doctor early in 1897. According to TCM theory, YYP can strengthen “Zheng Qi” and/or “blood Qi”, indicating it can enhance anti-injury and al serving role in the host against invading pathogens. It was revealed that extracts or constituents from some ingredient herbs of YYP, e.g. Dangshen (Radix Codonopsis), Baizhu (Rhizoma Atractylodis Macrocephalae), Gancao (Radix Glycyrrhizae), Rougui (Cortex Cinnamomi Cassiae), Shanyao (Rhizoma Dioscoreae Opposita), Longyanrou (Annona squamosa), Shudihuang (Radix Rehmanniae Preparata), Fuling (Poria), Huangqi (Radix Astragali Mongolici), Gouqizi (Fructus Lycii), Danggui (Radix Angelicae Sinensis), and Chenpi (Citrus reticulatae) that may have anti-inflammatory properties.

### Materials and Methods

#### Chemicals and reagents

Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and LPS (Escherichia coli O55: B5) were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT), Griess reagent (modified), Tween 20 and Bovine serum albumin (BSA) were purchased from Sigma Chemicals Ltd. (St. Louis, MO, USA). Antibodies against iNOS, COX-2, NF-κB p65, phospho-NF-κB p65 (Ser536), Akt, phospho-Akt (Ser473), inhibitor of κB α (IkBa), IkB kinase α/β (IKKα/β), and phospho-IKKα/β (Ser176/180), were from Cell signaling technology (Boston, MA, USA). β-actin and SP1 monoclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Ethanol (absolute, analytical grade), acetonitrile (ACN, HPLC grade) and formic acid (FA, HPLC grade) were purchased from RCI Labscan Limited (Bangkok, Thailand). Milli-Q water was prepared using a Milli-Q system (Millipore, MA, USA). 96-well plates and 60-mm-diameter culture dishes used in bioassays were from SPL Life Sciences (Gyeonggi-do, Korea).

#### Preparation of YYP extract

YYP was prepared with 12 herbs, including Dangshen (Radix Codonopsis), Baizhu (Rhizoma Atractylodis Macrocephalae), Gancao (Radix Glycyrrhizae), Rougui (Cortex Cinnamomi Cassiae), Shanyao (Rhizoma Dioscoreae Opposita), Longyanrou (Arrillus Longan), Shudihuang (Radix Rehmanniae Preparata), Fuling (Poria), Huangqi (Radix Astragali Mongolici), Gouqizi (Fructus Lycii), Danggui (Radix Angelicae Sinensis), and Chenpi (Citrus reticulatae) at the ratio of 51.7 : 40.7 : 28.2 : 7.8 : 6.3 : 4.7 : 3.1 : 3.1 : 1.5 : 1.0. All the herbs were provided by Wai Yuen Tong Medicine Company Limited and authenticated by the corresponding author. Voucher specimens of individual herbs were deposited at the Centre for Cancer and Inflammation Research, School of Chinese Medicine, Hong Kong Baptist University. The mixture of these herbs was ground and extracted with 95% ethanol. Briefly, 20 g powder of YYP was macerated with 200 mL 95% ethanol for 1 h at room temperature, and refluxed twice for 2 h. The extracts were then filtered, combined and evaporated under negative pressure in a rotary evaporator. The residue was lyophilized to obtain 4.47 g powder (yield: 22.35%), named as YYP for short. The extract was dissolved in DMSO (250 mg/mL), filtered through a 0.22 μm syringe and stored at 4 °C. The stock solution was diluted with DMEM to obtain a series of concentrations immediately before use.

#### HPLC analysis of YYP

To control the quality of YYP, HPLC analysis was using Agilent 1200 series LC system equipped with a quaternary pump, a diode array UV/vis detector (Agilent Technologies, Palo Alto, CA, USA), and a Grace Altima C18 column (250 mm x 4.6 mm, i.d. 5 μm; Agilent Technologies, Palo Alto, CA, USA) was performed to determine the glycyrrhizic acid content. The mobile phases were composed of 0.1% FA in water (A) and ACN (B). A gradient elution method was optimized as follows: 5%-25% B at 0-30 min, 25%-60% B at 30-60 min. The flow rate was 1.0 mL/min and an injection volume of sample was 10 μL in each assay. The detection wavelength was set as 250 nm.
Yin CL et al. / Research Article

Cell culture
The RAW 264.7 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM containing 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37 °C under a 5% CO₂ atmosphere.

Cell viability assay
The cytotoxic effect of YYP on RAW 264.7 cells was assessed using the MTT assay. RAW 264.7 cells were seeded (5 × 10⁴ cells per well) in 96-well plates for 24 h. Then the cells were pre-treated with YYP at various concentrations (100, 200, 300, 400 μg/mL) for 1 h, and cultured for another 24 h in the absence or presence of LPS (1 μg/mL) at 37 °C. After the addition of MTT solution (5 mg/mL) to each well, the cells were incubated for 2 h and then the medium was discarded. 100 μL DMSO was added to each well for dissolving the crystals. The absorbance of the samples was measured at 570 nm using a microplate spectrophotometer. The results were expressed as the percentage of cell viability to corresponding vehicles.

NO production assay
NO production was determined by measuring the content of nitrite in the supernatant of RAW 264.7 cells using the Griess test. RAW 264.7 cells were seeded (1 × 10⁵ cells per well) in 96-well plates for 24 h. The cells were pre-treated with YYP at various concentrations (50, 100, 200, 300 μg/mL) for 1 h, followed by incubation with or without LPS (1 μg/mL) at 37 °C for another 24 h. 100 μL of supernatant was mixed with equal volume of Griess reagent, and incubated at room temperature for 10 min. Absorbance of each well at 540 nm was measured using a microplate spectrophotometer. Nitrite concentrations in the supernatants were calculated using the sodium nitrite standard curve.

Enzyme-linked immunosorbent assay (ELISA) of PGE₂
ELISA kit purchased from Enzo Life Sciences (Farmingdale, NY, USA) was used to measure the concentrations of PGE₂ in the cell culture supernatants. RAW 264.7 cells were seeded (1 × 10⁵ cells per well) in 96-well plates for 24 h. After the pre-treatment with YYP at various concentrations (100 and 300 μg/mL) for 1 h, the cells were incubated with or without LPS (1 μg/mL) at 37 °C for another 24 h. Then the supernatants were collected and analyzed for PGE₂ production according to the manufacturer’s instructions.

Real-time polymerase chain reaction (RT-PCR) analysis
RAW 264.7 cells pretreated with YYP (100 and 300 μg/mL) for 1 h were incubated with or without LPS (1 μg/mL) at 37 °C for 6 h. The cells were collected and the total RNA was isolated with Trizol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer’s instructions. Quantitative RT-PCR was conducted as previously reported. The primers designed are as follows: iNOS (Sense 5′-AGCAACTCTGCTGTTGTGTT-3′ and anti-sense 5′-TCTTCAGAGTCTGCCATTG-3′), COX-2 (Sense 5′-CTG-GAACATGGACTCTCAGTTGG-3′ and anti-sense 5′-AGGCCCTTTGCGCAGTGTG-3′), and TNF-α (Sense 5′-ATGAGAAGTCCCAATGCGG-3′ and anti-sense 5′-CTCCATTTGGTTGTTGGC-3′). A PCR reaction performed with primers of GAPDH (Sense 5′-GGGCTTCCGTGTTCC-3′ and anti-sense 5′-TGCCCTGCTTACACCTTC-3′) was used to normalize the RNA contents of all samples. RT-PCR was conducted with SYBR green reaction mixture in the ABI 7500 Fast Real-time PCR System (Applied Biosystems, Waltham, MA, USA).

Western blot analysis
RAW 264.7 cells were seeded (1 × 10⁵ cells per well) in 60-mm-diameter culture dishes for 24 h. After the pre-treatment with YYP at various concentrations for 1 h, the cells were incubated with or without LPS (1 μg/mL) at 37 °C for indicated times. Then the cells were harvested and lysed as previously reported to obtain the total cellular and nuclear proteins. Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) was employed to quantify the protein concentrations of samples according to the manufacturer’s protocol. The proteins were then electrophoresed on a 10% SDS-Polyacrylamide gel and transferred to nitrocellulose membrane. After blocking with 5% (w/v) non-fat milk in Tris-buffered saline (10 mM NaCl, 20 mM Tris-HCl, pH 7.4) containing 0.1% Tween 20 buffer for 1 h, membranes were incubated at 4 °C overnight with specific primary antibodies. The membranes were then washed in Tris-buffered saline containing 0.1% Tween 20 three times and incubated with secondary antibodies for 1 h at room temperature. Specific proteins were visualized using the enhanced chemiluminescence ECL detection kit (Invitrogen, Carlsbad, CA, USA). The relative protein levels were quantified by Image J software.

Statistical analysis
All data from independent experiments were expressed as mean ± standard deviation (x ± s) and analyzed by one-way analysis of variance followed by Dunnett’s post hoc test using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). A P < 0.05 was considered statistically significant.

RESULTS
YYP inhibits LPS-induced production of NO and PGE₂ in RAW 264.7 macrophages
In order to ensure the stability of YYP and the repro-
ducibility of present experiments, an anti-inflammatory compound in YYP, glycyrrhizic acid, was identified and quantified using the HPLC analysis (Figure 1A). The content of glycyrrhizic acid in YYP was 0.56%. To determine the sub-lethal concentrations of YYP, MTT assays were conducted in RAW 264.7 macrophages. YYP showed no obvious cytotoxic effects on RAW 264.7 cells at concentrations varying from 100 to 400 μg/mL after a 24-h incubation in the absence or presence of LPS (1 μg/mL) (Figure 1B). Sub-lethal concentrations of YYP were used in the following experiments. As depicted in Figure 2, the levels of two inflammatory mediators, NO and PGE$_2$, were markedly increased upon LPS stimulation, compared to vehicle groups. YYP treatment significantly inhibited LPS-induced production of NO and PGE$_2$ in a dose-dependent manner (Figure 2A and 2B). Morphological changes (dendritic-like transformation) in LPS-stimulated RAW 264.7 cells were also reversed by YYP treatment (Figure 2C).

**YYP inhibits LPS-induced elevation of protein and mRNA levels of iNOS, COX-2 and TNF-α in RAW 264.7 macrophages**

Western blot analysis was applied to examine protein levels of two inflammation-related molecules, iNOS and COX-2, in LPS-stimulated RAW 264.7 macrophages. As shown in Figure 3, after 24 h of stimulation with LPS, protein levels of iNOS and COX-2 were up-regulated obviously, compared to vehicle groups. Treatment with YYP inhibited the up-regulation of iNOS and COX-2 in a concentration-dependent manner. RT-PCR analyses were performed to determine whether YYP inhibits LPS-induced production of iNOS and COX-2 at the transcriptional stage. Results in Figure 4A and 4B showed that mRNA levels of iNOS and COX-2 in RAW 264.7 cells were up-regulated markedly after a 6-h incubation with LPS. YYP treatment dose-dependently suppressed the LPS-induced elevation of mRNA levels of iNOS and COX-2. LPS-induced up-regulation of mRNA level of TNF-α, a...
and 0.01, compared with vehicle group; *P < 0.05 and **P < 0.01, compared with LPS group.

Yin CL et al. / Research Article

Figure 2 YYP inhibits LPS-induced production of NO and PGE in RAW 264.7 macrophages
A: effect of YYP on NO production in LPS-stimulated RAW 264.7 cells; B: effect of YYP on PGE production in LPS-stimulated RAW 264.7 cells; C: effect of YYP on morphology of LPS-stimulated RAW 264.7 cells. C1: vehicle group; C2: LPS group; C3: YYP 100 µg/mL-LPS group; C4: YYP 300 µg/mL-LPS group. Cells were incubated with the indicated concentrations of YYP for 1 h and then stimulated with or without LPS (1 µg/mL) for 24 h. NO and PGE were measured as described in the Materials and Methods section. Representative immunoblotting images of RAW 264.7 cells were obtained with 200 x objective using a video camera attached to an inverted microscope (Leica DMI3000 B). Data in A and B are shown as mean ± standard deviation from three independent experiments. NO: nitric oxide; PGE: prostaglandin E2; YYP: Young Yum pill; LPS: lipopolysaccharide. *P < 0.01, compared with vehicle group; *P < 0.05 and **P < 0.01, compared with LPS group.

Figure 3 YYP inhibits LPS-induced elevation of protein levels of iNOS and COX-2 in RAW 264.7 macrophages
A: effect of YYP on protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells; B: relative protein level of iNOS; C: relative protein level of COX-2. Cells were treated with YYP with the indicated concentrations for 1 h and then stimulated with or without LPS (1 µg/mL) for 24 h. iNOS and COX-2 protein levels were determined by Western blotting. Representative immunoblotting images are shown in A. The relative protein levels (iNOS/β-actin and COX-2/β-actin) in B and C were quantified by Image J software. Data are presented as mean ± standard deviation from three independent experiments. iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; YYP: Young Yum pill; LPS: lipopolysaccharide. *P < 0.01, compared with vehicle group; **P < 0.01 and ***P < 0.05, compared with LPS group.

DISCUSSION

NO, PGE₂, and TNF-α play vital roles in inflammatory responses. We found YYP could effectively suppress LPS-induced production of these inflammatory mediators in RAW 264.7 macrophages. NO production is mainly catalyzed by the enzyme iNOS, while PGE₂ is predominantly converted from arachidonic acid by the inducible enzyme COX-2. YYP was also found to decrease protein and mRNA levels of iNOS and COX-2, contributing to the inhibitory effects of YYP on NO and PGE₂ production, respectively.
NF-κB, a transcription factor participated in inflammatory reaction, was shown to regulate inflammatory mediators at the transcriptional level (Figure 7). Specifically, in un-stimulated cells, NF-κB mainly exists in cytoplasm and is bound to IkBα, which prevents it from entering the nuclei. Stimulation of cells with LPS leads to phosphorylation and activation of IKKα/β. Activation of IKKα/β subsequently phosphorlats IkBα, and causes its rapid degradation by proteasomes. Then NF-κB is activated or phosphorylated, resulting in its translocation into the nucleus, where it binds to specific sequences of promoter regions of genes and initiates the expression of various inflammation-related molecules (iNOS and COX-2) and inflammatory mediators (NO, PGE2 and TNF-α). In our study, YYP decreased IKKα/β phosphorylation, IkBα degradation, as well as NF-κB p65 phosphorylation and nuclear localiza-
Figure 6 YYP regulates the molecules involved in the NF-κB pathway in LPS-stimulated RAW 264.7 macrophages
A: effect of YYP on protein levels of the molecules involved in the NF-κB pathway; B: relative protein level of p-Akt/Akt; C: relative protein level of p-IKKα/β; D: relative protein level of p-p65/p65; E: relative protein level of IκBa/β-actin. Cells were treated with YYP at the indicated concentrations for 1 h and then stimulated with or without LPS (1 μg/mL) for 15 min. Protein levels were determined by Western blotting. Representative immunoblotting images are shown in A. The relative protein levels (p-Akt/Akt, p-IKKα/β/IκKα/β, p-p65/p65 and IκBa/β-actin) in B-E were quantified by Image J software. Data are shown as mean ± standard deviation from three independent experiments. p-Akt: phosphorylated Akt (Ser473); p65: nuclear factor-kappa B p65 subunit; p-p65: phosphorylated NF-κB p65 (Ser536); IκBa: inhibitor of κB α; IκKα/β: IκB kinase α/β; p-IKKα/β: phosphorylated IκKα/β (Ser176/180); YYP: Young Yumpill; LPS: lipopolysaccharide. *p < 0.01, compared with vehicle group; †p < 0.05 and ‡p < 0.01, compared with LPS group.

Figure 7 Inhibitory effects of YYP on the NF-κB-regulated inflammatory mediators in LPS-stimulated RAW 264.7 macrophages

REFERENCES


