International Journal of Biological Macromolecules 59 (2013) 357–362

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Effects of carboxymethylpachymaran on signal molecules in chicken immunocytes

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ARTICLE INFO

Article history:
Received 15 February 2013
Received in revised form 20 April 2013
Accepted 27 April 2013
Available online 7 May 2013

Keywords:
Carboxymethylpachymaran
Lymphocytes
Signal molecules

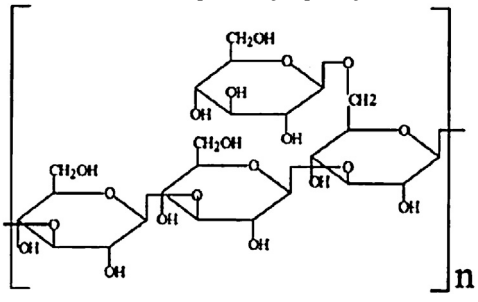
ABSTRACT

The study was carried out to investigate the immunomodulation mechanism of carboxymethylpachymaran (CMP). Chicken splenic lymphocytes were cultured in medium alone or with CMP at the final concentration of 50 mg/L, 100 mg/L, 200 mg/L or 400 mg/L *in vitro* for 4 h, 8 h, 12 h or 24 h, respectively. The supernatants at different culture periods were analyzed for changes in levels of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), thromboxane B_2 (TXB_2) and nitric oxide (NO). The cells were collected to determine contents of oxidized glutathione (GSSG), reduced glutathione (GSH), cyclic AMP (cAMP) and cyclic GMP (cGMP). The results showed that CMP increase the values of NO, 6-keto-$PGF_{1\alpha}$, TXB_2, and the ratio of 6-keto-$PGF_{1\alpha}$ to TXB_2 in supernatants. The contents of intracellular GSH, cAMP, cGMP and the ratio of cAMP to cGMP were increased in the cells treated with CMP. The results suggested that CMP enhanced immune functions by increasing the contents of GSH and by regulating arachidonic acid signal transduction systems in chicken splenic lymphocytes. The signal pathway of NO-cGMP plays an important role in CMP-induced activation of chicken splenic lymphocytes.

1. Introduction

Lymphocytes need to be activated in order to carry out their function, at the molecular level this means receiving a message from outside the cell via interaction with a cell surface receptor. This signal is then passed through the cytoplasm to the nucleus (signal transduction) to induce the gene transcription required for cell proliferation and synthesis and release of effectors molecules, e.g., cytokines and antibodies [1]. Many plant-derived polysaccharides are studied in the biomedical area because of their immunostimulating activities. Some polysaccharides from plants exhibit various biological actions involved in the activation of human T lymphocytes and mouse B lymphocytes, as well as monocytes and macrophages [2–6].

In traditional Chinese herbal medicine, *Poria cocos* is claimed to be effective in treating edema and clear febrile illnesses [7]. The polysaccharide of *P. cocos* have long been proven to exhibit strong anti-inflammatory effects, antitumor and immunomodulatory activities [8,9]. However, the main chemical component of *P. cocos* is a water-insoluble β-(1→3)-D-glucan which hardly shows bioactivities [10]. Alternatively, native insoluble polysaccharides can be chemically modified for enhancing their bioactivities [11,12]. It has been reported that carboxymethylpachymaran (CMP), carboxymethylated β-(1→3)-D-glucan from pachymaran, had good water solubility and enhanced anti-tumor activity [13]. The present study was undertaken to investigate the role of CMP in activation of chicken splenic lymphocytes.



2. Materials and methods

2.1. CMP and reagents

CMP was prepared as previously described [14]. A water-insoluble (1→3)-β-D-glucan termed as pachymaran was extracted with 0.5 M NaOH aqueous solution from sclerotium of *P. cocos*. Six hundred milligram of pachymaran was suspended in a solution of 10 mL of 20% NaOH and 25 mL isopropanol in an ice bath with stirring for 3 h. Then, a solution of 5.25 g chloroacetic acid, 10 mL of

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http://dx.doi.org/10.1016/j.ijbiomac.2013.04.071

20% NaOH and 25 mL isopropanol were slowly added with stirring. The reaction was continued at room temperature for 3 h and then at 60 °C for 1.5 h. After the solution was cooled to the room temperature, 0.5 M HCl was added to adjust pH to 7 and then dialyzed by a regenerated cellulose tube (M_w cut-off 8000) against tap water for 7 days and distilled water for 4 days, respectively. The resulting solution was concentrated by rotary evaporator at reduced pressure below 40 °C. Finally, the carboxymethylated (1→3)-β-D-glucan was lyophilized by using a lyophilizer (CHRIST Alpha1-2, Germany) to obtain a white powder. Total sugar content was more than 65%, estimated by the phenol–sulfuric acid assay using D-glucose as the standard [15]. Endotoxin was assayed under endotoxin free experimental conditions using a Limulus amebocytes lysate (LAL) pyrogen kit (Zhanjiang Adc Biological). The experiments were carried out according to the manufacture's protocol: 100 μL of standards, CMP or controls were mixed with 100 μL of LAL reagent and incubated for 1 h at 37 °C. Each tube was then examined for gelation. The quantity of endotoxin in CMP was ≤0.005 ng/mg. CMP was dissolved in distilled water in a concentration of 10 g/L and then filtered through a 0.22-μm filter and stored at 4 °C. It was further diluted to indicated concentration with RPMI-1640 medium in tissue plate separately (50 mg/L, 100 mg/L, 200 mg/L and 400 mg/L). RPMI-1640 was obtained from Gibco Laboratories. Fetal calf serum (FCS) was from Sijiqing Institute of Biomaterials, Hangzhou, China. *N*-(1-naphthyl)-ethyldiamine dihydrochloride and sulfanilamide were purchased from ICN Biomedicals, USA. All other chemicals used were ultrapure or analytic grade.

The chemical structure of carboxymethylpachymaran was nonselective and occurred mainly at C-6 position and secondly at C-4 and C-2 position of the (1→3)-β-D-glucan [16]. The previous work indicated that CMP was pure polysaccharide consisted of carboxymethyl glucose only, without evidence showing the existence of nucleic acid or protein, CMP was β-type glucosan with its glucosidic bond is connected by β(1→3) and β(1→6). CMP was glucopyranose and its molecular weight was about 4.2×10^4 Da [17].

2.2. Splenic lymphocytes isolation and culture

SPF Leghorn chickens at 30 days old (purchased from Animal Center of Guangxi University, Guangxi, China) were sacrificed by intravenous barbiturates. Spleens were collected aseptically and rinsed with Ca^{2+}- and Mg^{2+}-free PBS (717 mmol/L K_2HPO_4, 283 mmol/L KH_2PO_4, pH 7.2), chopped into small pieces and passed through a 10 μm nylon mesh, then diluted with equal volume of Hanks' solution and carefully layered on the surface of lymphocyte separation medium. After centrifugation at 1500 rpm for 10 min at 4 °C, a white cloud-like band containing lymphocytes was collected and washed twice with RPMI 1640 media without fetal bovine serum. Finally, the splenic lymphocytes were resuspended to 5×10^6 mL^{-1} with RPMI 1640 medium supplemented with 10% FCS (56 °C for 30 min), 100 units/mL benzylpenicillin sodium, 100 μg/mL streptomycin, 2 mM glutamine and 50 μM β-mercaptoethanol in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cell viability was estimated according to the trypan blue exclusion criteria and the purity of splenic lymphocytes was higher than 95% as described [18].

2.3. Nitrite measurement

Chicken splenic lymphocytes were set up in 24-well plates (2×10^6 mL^{-1}/well) and incubated for 4 h, 8 h, 12 h or 24 h in phenol-red free RPMI-1640 tissue culture medium in the presence of CMP (0 mg/L, 50 mg/L, 100 mg/L, 200 mg/L or 400 mg/L). NO concentration in the cell-free culture supernatant was measured by a spectrophotometric assay based on the Griess reaction as described [19,20]. Briefly, a volume 100 μL of the culture supernatant was mixed with an equal volume of Griess reagent [one part of 0.1% (w/v) naphthylethylenediamine dihydrochloride in distilled water plus one part of 1% (w/v) sulphanilamide in 5% (v/v)·H_3PO_4] at room temperature. Fifteen minutes later, the absorbance was determined with an automatic ELISA plate reader at 540 nm. The NO concentration was determined by a standard curve of $NaNO_2$.

2.4. Radioimmunoassay for cAMP and cGMP contents

Chicken splenic lymphocytes were set up in 24-well plates (5×10^6 mL^{-1}/well) and incubated for 4 h, 8 h, 12 h or 24 h in phenol-red free RPMI-1640 tissue culture medium in the presence of CMP (0 mg/L, 50 mg/L, 100 mg/L, 200 mg/L or 400 mg/L), then lymphocytes were collected and subjected to centrifugation. The cell pellets were treated with 1 mL 50 mmol/L acetic acid (pH 4.75) and stored at −20 °C until analysis. The cells, broken by repeated freezing and thawing, were centrifuged at 3000 rpm for 15 min at 4 °C. The supernatants were measured for cGMP and cAMP by radioimmunoassay using a commercially available kit (Beijing North Institute of Biological Technology, Beijing, China) as reported previously [21]. Briefly, portions of samples (100 μL/sample) were acetylated and then incubated with 100 μL ^{125}I-cGMP/^{125}I-cAMP (20,000 cpm/100 μL) and 100 μL anti-cGMP/anti-cAMP antibody at 4 °C for 24 h. Rabbit serum (100 μL) and goat anti-rabbit IgG (100 μL) were added to the mixture for 12 h at 4 °C, then the mixture was centrifuged at 3000 rpm and 4 °C for 20 min. Radioactivity of the deposits was measured by use of a multi-well gamma counter (Ruikang hospital, Guangxi, China).

2.5. Reduced (GSH) and oxidized (GSSG) glutathione assay

Chicken splenic lymphocytes were set up in 24-well plates (5×10^6 mL^{-1}/well) and incubated for 4 h, 8 h, 12 h or 24 h in phenol-red free RPMI-1640 tissue culture medium in the presence of CMP (0 mg/L, 50 mg/L, 100 mg/L, 200 mg/L or 400 mg/L), then lymphocytes were collected and subjected to centrifugation. The cell pellet was treated with 1 mL 50 mmol/L acetic acid (pH 4.75) and stored at −20 °C until analysis. The cells, broken by repeated freezing and thawing, homogenized with 10% trichloroacetic acid (TCA). After centrifugation at 3000 rpm and 0 °C for 10 min, the supernatant was used for the GSH and GSSG assay as reported previously [22]

2.6. Radioimmunoassay for 6-keto-$PGF_{1\alpha}$ and TXB_2 content

Chicken splenic lymphocytes were set up in 24-well plates (5×10^6 mL^{-1}/well) and incubated for 4 h, 8 h, 12 h or 24 h in phenol-red free RPMI-1640 tissue culture medium in the presence of CMP (0 mg/L, 50 mg/L, 100 mg/L, 200 mg/L or 400 mg/L). The supernatants were measured for TXB_2 and 6-keto-$PGF_{1\alpha}$ by radioimmunoassay using a commercially available kit (Beijing North Institute of Biological Technology, Beijing, China) as reported previously [23,24]. Radioactivity of the deposits was measured by use of a multi-well gamma counter (Ruikang hospital, Guangxi, China).

2.7. Statistical analysis

Quantitative data were expressed as mean ± S.D. All statistical comparisons were carried out by means of one-way ANOVA test followed by Tukey's test. *P*-values less than 0.05 were considered