International Journal of Biological Macromolecules 69 (2014) 382–387

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Polyphenols from blueberries modulate inflammation cytokines in LPS-induced RAW264.7 macrophages

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ARTICLE INFO

Article history:
Received 4 April 2014
Received in revised form 15 May 2014
Accepted 19 May 2014
Available online 3 June 2014

Keywords:
Blueberries
Polyphenols
Anti-inflammation

ABSTRACT

Polyphenols including 3-glucoside/arabinoside/galactoside-based polymers of delphinidins, petunidins, peonidins, malvidins and cyanidins are one type of biological macromolecules, which are extraordinarily rich in blueberries. Anti-inflammatory activity of blueberry polyphenols (BPPs) was investigated by using lipopolysaccharide (LPS) induced RAW264.7 macrophages. The results showed that BPPs suppressed the gene expression of IL-1β (interleukin-1β), IL-6 and IL-12p35. The inhibition effect on IL-1β and IL-6 mRNA was most obvious at the concentration of 10–200 μg/mL BPPs. But the inhibition effect on IL-12p35 mRNA was increased with the increasing concentration of BPPs. When fixed at 100 μg/mL BPPs, the most significant inhibition on IL-1β, IL-6 and IL-12p35 mRNA expression was detected at 12–48 h. In conclusion, BPPs exhibit anti-inflammation activity by mediating and modulating the balances in pro-inflammatory cytokines of IL-1β, IL-6, and IL-12.

1. Introduction

Macrophages are the first line of host defenses against bacterial infection and cancer growth and thus play an important role in the initiation of adaptive immune responses [1]. Macrophages were stimulated by bacterial products including lipopolysaccharides (LPS) and muramyl dipeptide and release several inflammatory cytokines, interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor-α (TNF-α), and nitricoxide (NO), all of which directly induce tumoricidal or inflammatory activity in macrophages [2]. IL-1 has direct in vitro cytostatic and cytocidal effects; IL-6 is also considered as a major immune and inflammatory mediator; IL-12 produced by macrophages enhances T-cell responsiveness. The above three cytokines are related to each other in that they are coordinately released from activated macrophages, and that IL-1 can induce IL-6. However, IL-6 does not induce IL-1, but rather suppresses their production by macrophages [3]. Chronic inflammation is a hallmark of several pathologies, such as rheumatoid arthritis, inflammation bowel disease, atherosclerosis and cancer. Acute inflammation is a protective response of the host against irritation, injury and infection. Its primary function is to induce the secretion of several pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 and IL-12, while low levels of cytokines with anti-inflammatory effects such as IL-4, IL-10 and transforming growth factor-β (TGF-β) are produced by the insulted tissue or cells. However, when it becomes chronic inflammation can lead to cancer, diabetes, pulmonary, cardiovascular and autoimmune diseases. In fact, the main causes of inflammation are not well understood, but imbalances in pro-inflammatory TNF-α, interferon-γ (IFN-γ), IL-1, IL-6, and IL-12 and anti-inflammatory cytokines including IL-4, IL-10, and IL-11 are thought to play a central role in mediating and modulating inflammation [4].

Plant polyphenols are a group of chemicals that have more than one phenol ring per molecule. They are found in grapes, apples, berries, tea, coffee, and also in other fruits and vegetables that represent an important part of the human diet. The structures of natural polyphenols vary from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins [5]. These compounds contain sufficient hydroxyls and other suitable groups such as carboxyls to form strong complexes with proteins and other macromolecules [6]. Some researchers have reported that high consumption of polyphenols has protective effects against cancer and inflammatory diseases [7]. The

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http://dx.doi.org/10.1016/j.ijbiomac.2014.05.071
0141-8130/

anti-inflammatory effect of polyphenols has been attributed primarily to their antioxidant activity because they were known to scavenge and prevent the formation of reactive oxygen (ROS) and nitrogen species [8,9], which are important hallmarks of inflammation. The cell lines have been widely used in the mechanism and pharmacology of potential anti-inflammatory polyphenols from plants [10,11].

Blueberry is an increasingly important commercial crop and widely planted in many regions of the world. Many reports have indicated that blueberry fruits have a wide range of beneficial properties to health, such as anti-inflammatory [11,12], antimicrobial [13], anticancer [14], and antioxidant [15] activities. In blueberries many bioactive compounds, such as polyphenols, terpenes, and dietary fiber are found. Polyphenols are widely studied today because of their strong antioxidant health benefits. Previous reports pointed out that polyphenols from blueberries were 3- glucoside/arabinoside/galactoside-based polymers consisting of delphinidins, petunidins, peonidins, malvidins and cyanidins [16,17], and those polymers were considered as one type of biological macromolecules.

Numerous investigators reported that plant derivatives such as phenolic compounds and flavonoids exhibit anti-inflammatory activity by modulating the expression levels of various cytokines. However, the anti-inflammatory activity of blueberry polyphenols (BPPs) was little known. Especially, there were few reports on the verification ofthe anti-inflammatory activity and mechanisms by using the cell model of RAW264.7. The anti-inflammatory mechanisms of polyphenols were complex. The primary effect is to induce the secretion and gene expression of pro-inflammatory and anti-inflammatory effectors and enzymes, and to modulate many signal transduction pathways. But, in the present study, we first extracted and purified BPPs, and determined the main individual compounds by HPLC, then further preliminarily predicated the anti-inflammatory mechanisms of BPPs which activated the RAW264.7 cells. We mainly investigated the effects of BPPs on several pro-inflammatory cytokines' (IL-1β, IL-6, IL-12) gene expression in LPS-induced RAW264.7 macrophages.

2. Materials and methods

2.1. Reagents

Standard samples of Dp-Glc (Delphinidin-3-glucoside), Dp-Gal (Delphinidin-3-galactoside), Cy-Gal (Cyanidin-3-galactoside), Dp-Ara (Delphinidin-3-arabinoside), Cy-Glc (Cyanidin-3-glucoside), Pt-Glc (Petunidin-3-glucoside), Pn-Gal (Peonidin-3-galactoside), Pn-Glc (Peonidin-3-glucoside), Mv-Gal (Malvidin-3-galactoside), Mv-Glc (Malvidin-3-glucoside) and Mv-Ara (Malvidin-3-arabinoside) were purchased from Tauto Company (Shanghai, China). Fetal bovine serum (FBS), cell culture medium (DMEM-2), and penicillin–streptomycin were purchased from Gibco (USA), lipopolysaccharide (LPS) was purchased from Sigma (USA), and phosphate buffer solution (PBS) from Hyclone (USA). Cell culture dishes and multi-well plates were supplied by Nunc (Denmark). HP-20 resin, acetonitrile, formic acid, ethanol, and tea polyphenols (TP) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Trizol was purchased from Invitrogen (USA). ELISA Kits for IL-1β, IL-6, and IL-12P35 detection were from Shanghai Sangon Biotech Co. Ltd. (China).

2.2. Preparation of blueberry polyphenols (BPPs)

The preparation of crude BPPs was performed as our previous report [18]. The concentrated extracts were applied to a HP-20 resin column (2.5×60 cm) and the bound materials were eluted with 80% ethanol. The collected elution was concentrated to 1/5 volume. The concentrated elution was dried with a freeze dryer (FD-1C-50, Boyikang Comp., Beijing, China). The above-dried BPPs were dissolved in PBS and filtered through a 0.22 μm filter before being used.

2.3. HPLC analysis

The purified BPPs were analyzed by using an Agilent 1200 series HPLC (Agilent Technologies Ltd.), equipped with a binary pumping system. In accordance with Bijak et al. [19], samples were injected into a Zorbax C18 column (4.6×150 mm, particle size 5 μm) (Agilent). The mobile phase consisted of water/formic acid (90:10, v/v) (A) and water/acetonitrile/formic acid (40:50:10, v/v/v) (B). The flow rate was 1 mL/min with the following gradient program: 0 min: 88% A + 12% B, 26 min: 70% A + 30% B, 40–43 min: 0% A + 100% B, and 48–50 min: 88% A + 12% B. Major individual compounds of BPPs were analyzed at a wavelength of 520 nm.

2.4. Cell culture

RAW264.7 cell lines (No. GDC143) were friendly gifts from the China Center of Type Culture Collection (CCTCC, Wuhan, China). RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in a 5% CO_2 humidification atmosphere (Thermo Scientific BBD6220, Germany). Cells were inoculated in a 25 mL cell plate in a total volume of 7 mL at a concentration of 1.2×10^6 cells/mL with complete media containing LPS (1 μg/mL), and the culture was incubated for 24 h at 37 °C in a 5% CO_2. After 24 h the culture was treated with LPS, and the media were removed and adherent cells were washed twice with PBS. Adherent macrophages (1.2×10^6 cells/mL) were placed in cell culture dishes and incubated in complete DMEM alone (control group, CK) or in media containing BPPs, and 100 μg/mL TP as a positive control. Macrophages (1.2×10^6 cells/mL) were cultured in the presence of different concentrations (10, 100, 200, 400 μg/mL) of BPPs in a 25 mL cell plate in a total volume of 7 mL up to 48 h, or cultured for different time (6, 12, 24, 48, 72 h) periods in the presence of BPPs (100 μg/mL). Finally the cells were collected from the treatments.

2.5. Real time RT-PCR for IL-1β, IL-6 and IL-12p35 mRNA expression

Total RNA was isolated from the control and treated cells using Trizol. The quantity of total RNA was determined by a spectrophotometer using the absorbance at A260/A280 nm. cDNA was generated using the High Capacity RNA-to-cDNA Kit (Invitrogen, USA). cDNA was used as a template for real-time PCR in triplicates with Fast SYBR Green Master Mix and gene-specific primers. PCR analysis was performed on a Stepone Real-time PCR system (Applied Biosystems). The upstream and downstream primer sequences were as follows: for IL-1β: F-5′-AGA TAG AAG TCA AGA GCA AAG TGG A-3′ and R-5′-TGG GGA AGG CAT TAG AAA CAG-3′ (expected product 186 bp); for IL-6: F-5′-CTG GGA AAT CGT GGA AAT GAG-3′ and R-5′-GAC TCT GGC TTT GTC TTT CTT GTT A-3′ (expected product 247 bp); for IL-12p35: F-5′-TTG ATG ATG ACC CTG TGC CT-3′ and R-5′-GAT TCT GAA GTG CTG CGT TGA-3′ (expected product 91 bp); for β-actin: F-5′-GTG CTA TGT TGC TCT AGA CTT CG-3′ and R-5′-ATG CCA CAG GAT TCC ATA CC-3′ (expected product 174 bp). Cycling was initiated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 40 s, and 72 °C for 1 min. The relative expression of each gene was calculated using the comparative threshold cycle method normalized to β-actin.