International Immunopharmacology 42 (2017) 157–167

Contents lists available at ScienceDirect

International Immunopharmacology

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

Gamma irradiation enhanced Tollip-mediated anti-inflammatory action through structural modification of quercetin in lipopolysaccharide-stimulated macrophages

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ARTICLE INFO

Article history:
Received 10 August 2016
Received in revised form 25 October 2016
Accepted 25 November 2016
Available online 1 December 2016

Keywords:
Quercetin
Gamma irradiation
Toll-interacting protein
Mitogen-activated protein kinases
Nuclear factor-κB

ABSTRACT

The changes in molecular structure and anti-inflammatory action of a gamma-irradiated quercetin were examined. Quercetin was gamma-irradiated at doses of 0, 15, 30, 50, 100 and 150 kGy, which induced new radiolytic peaks (the highest radiolytic peak at a dose of 30 kGy). Treatment of intact- and gamma-irradiated quercetin did not induce a significant cellular toxicity of macrophages at concentrations ranging from 12.5 to 50 μM. Treatment of LPS-stimulated macrophages with gamma-irradiated quercetin (30 kGy) showed a higher inhibitory action than intact-quercetin groups in the excessive expression of inducible nitric oxide synthases-mediated nitric oxide, prostaglandin E2, pro-inflammatory cytokines level, such as tumor necrosis factor-α, interleukin-6 and interleukin-1β, reactive oxygen species, as well as cell surface molecules (CD80, CD86, and MHC class I/II). The inhibition of LPS-stimulated pro-inflammatory mediators was mediated through a suppression of mitogen-activated protein kinases and nuclear factor-κB pathways. In addition, gamma-irradiated quercetin (30 kGy) markedly elevated the expression of the Toll-interacting protein compared to intact-quercetin. The inhibitory action of intact- and gamma-irradiated quercetin on the production of IL-6 and TNF-α was not observed in the down-regulation of Tollip. Therefore, these findings represent new insights into the understanding of the changes in molecular structure and the physiological properties of natural products through the application of radiation technology.

1. Introduction

Flavonoids are widely present in all plants, such as fruits and vegetables, and the biological activities of flavonoids have been regarded as much scientific interests because of their health-beneficial effects for both prevention and treatment of various inflammation-related chronic diseases [1–4]. These aforementioned references have strongly suggested that higher flavonoid-rich diets may be believed to act as a promising approach to counteract risk for a wide variety of inflammatory diseases [5,6]. Quercetin, one of the most commonly distributed flavonoids in fruits and vegetables, has been of great scientific interest over the years, and the dietary supplementation of quercetin has been given to many benefits and medicinal properties, such as anti-inflammatory, anti-cancer, anti-viral, anti-obesity, and anti-oxidative effects [7,8]. In particular, inflammatory response is considered as a critical factor of chronic joint diseases underling a wide variety of human pathological processes, such as cancer, obesity, cardiovascular diseases, and diabetes [5,9,10]. The inflammatory response is caused by various pro-inflammatory mediators (e.g., inducible nitric oxide synthases (iNOS), nitric oxide (NO), prostaglandin E2 (PGE_2), cyclooxygenase (COX), and cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6), and the pro-inflammatory mediators are ultimately caused by two separate signaling pathways including nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPKs), as well as reactive oxygen species (ROS) generation [11–14]. It has recently become clear that quercetin is believed to play a role in both

Abbreviations: HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LPS, lipopolysaccharide; Tollip, toll-interacting protein; TLR, toll-like receptor; MHC, major histocompatibility complex; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; TNF-α, tumor necrosis factor-α; IL, interleukin; MAPK, mitogen-activated protein kinase; COX, cyclooxygenase; iNOS, nitric oxide synthase; NO, nitric oxide; ELISA, enzyme-linked immunosorbent assay; PGE_2, prostaglandin E2; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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http://dx.doi.org/10.1016/j.intimp.2016.11.030
1567-5769/

prevention and treatment of the inflammatory diseases through the suppression of the pro-inflammatory mediators as mentioned above [15,16]. Interestingly, our experimental data have demonstrated that the anti-inflammatory action of quercetin seems to be mediated through the expression of toll-interacting protein (Tollip), a negative regulator of toll-like receptor (TLR) signaling [17].

Gamma irradiation is well-known to be used to improve the safety and shelf-life of foods by controlling pathogen-induced poisoning, and has also been found to have importance in the sterilization processes for medical supplies and beauty care industry [18,19]. Furthermore, the irradiation with gamma rays has been applied in the fields of the structural modification of several natural molecules, such as genistein, resveratrol, and apigenin, which is suggested to play a major role showing that gamma irradiation can be an effective tool for a reduction of toxicity and an increase of the physiological activities of biomolecules [20–22]. Although many mechanisms for the anti-inflammation action of quercetin have been proposed in previous studies, the mechanisms responsible for the changes in anti-inflammatory action induced by gamma-irradiated quercetin are still not established. Furthermore, there is no evidence yet supporting the scientific information with regard to the modification of the structural properties of gamma-irradiated quercetin.

Therefore, in the present study, we estimated the physic-chemical and physiological changes of gamma-irradiated quercetin to gain further insight into an anti-inflammatory agent in the functional foods and medical industry.

2. Materials and methods

2.1. Materials

Quercetin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was purchased from Invivogen (San Diego, CA, USA). The fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) kit was purchased from R&D Systems (Minneapolis, MN). iNOS polyclonal anti-body (Ab), COX-2 polyclonal Ab, anti-CD14 polyclonal Ab, anti-phosphorylated extracellular signal-regulated kinase (ERK)1/2 monoclonal Ab, anti-phosphorylated p38 monoclonal Ab, anti-NF-κB (p65) polyclonal Ab, anti-phosphorylated inhibitor of κB (Iκ B)-α monoclonal Ab, anti-Tollip monoclonal Ab, and horseradish peroxidase (HRP)-conjugated anti-goat donkey IgG Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse IgG Ab and HRP-conjugated anti-rabbit Ab were obtained from Calbiochem (San Diego, CA), and anti-β-actin monoclonal Ab (AC-15) was purchased from Sigma-Aldrich (St. Louis, MO). APC-conjugated monoclonal Ab to F4/80, phycoerythrin (PE)-conjugated monoclonal Ab to CD80, CD86, and major histocompatibility complex (MHC) class I/II were purchased from eBioscience (San Diego, CA). IL-6, IL-1β, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from BD Biosciences (San Diego, CA).

2.2. Preparation of gamma-irradiated quercetin

Quercetin was dissolved in methanol to obtain a concentration of 1 mg/mL for the studies on structural properties. The quercetin solution was irradiated at 0, 15, 30, 50, 100, and 150 kGy, in a cobalt-60 irradiator (point source AECL, IR-221, MDS Nordion International Co. Ltd., Ottawa, ON, Canada) with a 11.1 peta-becquerel (PBq) source strength and operated at a dose rate of 10 kGy/h. Dosimetry was performed using an alanine dosimeter with a 5-mm diameter (Bruker Instruments, Rheinstetten, Germany). The dosimeter was calibrated using an International Atomic Energy Agency (Vienna, Austria) standard. Methanol solution of gamma-irradiated quercetin was evaporated using a rotary evaporator (Tokyo Rikakikai Co. Ltd., Japan) for the studies of the physiological property, and then both intact- and gamma-irradiated quercetin powders (10 mg/mL) were dissolved in dimethyl sulfoxide (DMSO) and stored at −80 °C.

2.3. Structural modification of gamma-irradiated quercetin using high-performance liquid chromatography (HPLC) analysis

Structural modification of gamma-irradiated quercetin was analyzed using an HPLC system (Agilent 1100 series; Agilent Technologies, Inc., Santa Clara, CA, USA) with a diode array detector. The sample concentration for loading was 100 μg/mL, and the injection volume was 20 μL. Samples were diluted in ethanol. All tested solutions were filtered through 0.22-μm nylon membrane syringe filters before HPLC analysis. The brief conditions of the HPLC system are as follows: Reverse phase: Agilent Eclipse XDB–C18 column (5 μM pore size and length I.D., 4.6 mm × 150 mm); Mobile phase: A: ethanol; B: 0.1% formic acid. Flow rate: 0.8 mL/min, detector: UV – 280 nm. These analyses were conducted using an isocratic elution during 60 min with A–B (75–25).

2.4. Cell culture

RAW264.7 macrophage cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). RAW264.7 macrophage cells were maintained in DMEM (GIBCO, Carlsbad, CA, USA) containing 10% FBS (GIBCO), 100 U/mL penicillin and 100 U/mL streptomycin (complete medium) under humidified conditions at 37 °C and 5% CO_2 in an incubator.

2.5. Cell proliferation

Cell proliferation was examined using the EZ-Cytox cell viability kit (Daeil Laboratories, Seoul, Republic of Korea) according to the manufacturer's instructions. RAW264.7 macrophage cells were seeded in 96-well plates at a density of 2×10^4 cells/well in DMEM. After incubation at 37 °C for 4 h, the medium was replaced with DMEM containing intact- and gamma-irradiated quercetin (at concentrations of 12.5, 25, and 50 μM) for 24 h. EZ-Cytox kit reagent (10 μL) was added to each well, and the cells were incubated for 1 h. The optical density was measured at 450 nm in an automated micro-plate reader (Zenyth 3100; Anthos Labtec Instruments GmbH, Salzburg, Austria). The gamma-irradiated quercetin was added to cultures of RAW264.7 macrophage cells in 12-well plates (0.5×10^6 cells/well). To investigate the cytotoxic effect of intact- and gamma-irradiated quercetin in RAW264.7 macrophage cells, the pattern of cell death of RAW264.7 macrophage cells was analyzed after treatment with intact- and gamma-irradiated quercetin at concentrations of 12.5, 25, and 50 μM. After 24 h of treatment, harvested RAW264.7 macrophage cells were washed with a phosphate-buffered saline (PBS) and stained by FITC-Annexin V and propidium iodide (BD PharMingen, San Jose, CA, USA). Thereafter, the cytotoxicity of RAW264.7 macrophage cells was analyzed by fluorescence-activated cell sorter (FACS) Calibur flow cytometry (BD, Franklin Lakes, NJ, USA).

2.6. NO and PGE_2 production

The concentration of NO in a culture supernatant was determined by measuring its oxidation product, nitrite. In this experiment, the Griess method (developed in 1879) was employed to detect NO, which is based on the chemical diazotization reaction. RAW264.7 macrophage cells (2×10^4 cells/well) were cultured in a 96-well plate, and then incubated at 37 °C and 5% CO_2 in an incubator for complete adherence. Intact- and gamma-irradiated quercetin (at concentrations of 12.5, 25, and 50 μM) were added to each well, and incubated at a 37 °C incubator for 24 h. The supernatant of the cell culture medium was mixed with the Griess reagent for an assessment of NO production. $NaNO_2$ prepared with freshly prepared in deionized water was used as the control