Biochemical and Biophysical Research Communications 413 (2011) 318–324

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Role of metabolism by the human intestinal microflora in arbutin-induced cytotoxicity in HepG2 cell cultures

Tilak Khanal [a,1], Hyung Gyun Kim [a,1], Yong Pil Hwang [a], Min Jeong Kong [b], Mi Jeong Kang [b], Hee Kyung Yeo [d], Dong Hyun Kim [c], Tae Cheon Jeong [b,*], Hye Gwang Jeong [a,*]

[a] *Department of Toxicology, College of Pharmacy, Chungnam National University, Daejeon 305-764, South Korea*
[b] *College of Pharmacy, Yeungnam University, Gyeongsan 712-749, South Korea*
[c] *College of Pharmacy, Kyung Hee University, Seoul 130-701, South Korea*
[d] *Department of Food and Nutrition, Kyung Hee University, Seoul 130-701, South Korea*

ARTICLE INFO

Article history:
Received 16 August 2011
Available online 26 August 2011

Keywords:
Intestinal microflora
Arbutin
Hydroquinone
Cytotoxicity
Metabolism

ABSTRACT

A possible role for metabolism by the human intestinal microflora in arbutin-induced cytotoxicity was investigated using human hepatoma HepG2 cells. When the cytotoxic effects of arbutin and hydroquinone (HQ), a deglycosylated metabolite of arbutin, were compared, HQ was more toxic than arbutin. Incubation of arbutin with a human fecal preparation could produce HQ. Following incubation of arbutin with a human fecal preparation for metabolic activation, the reaction mixture was filter-sterilized to test its toxic effects on HepG2 cells. The mixture induced cytotoxicity in HepG2 cells in a concentration-dependent manner. In addition, the mixture considerably inhibited expression of Bcl-2 together with an increase in Bax expression. Likewise, activation stimulated cleavage of caspase-3 and production of reactive oxygen species in HepG2 cell cultures. Furthermore, induction of apoptosis by the intestinal microflora reaction mixture was confirmed by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling assay. Taken together, these findings suggest that the human intestinal microflora is capable of metabolizing arbutin to HQ, which can induce apoptosis in mammalian cells.

1. Introduction

Food components may be metabolized in the gut not only by digestive and intestinal mucosal enzymes but also by the resident bacteria that are abundant in the large intestine. The intestinal microflora is related to human health and disease in a multitude of ways [1]. For example, it is deeply involved in metabolic functions that result in the modulation of energy metabolism and absorption of nutrients. In addition, bacteria have trophic effects on the intestinal epithelium and the immune system that are protective against alien microbes. With regard to metabolic functions, the intestinal microflora is also able to produce toxic or carcinogenic metabolites or new metabolites that are not produced in host tissues [2].

Arbutin, a glycosylated form of hydroquinone (HQ), is found in a variety of food plants and their products, such as wine, coffee, wheat products, broccoli, and certain fruits. The highest exposure to HQ can be achieved by oral intake of arbutin-rich foods. For example, pears reportedly contain up to 4.8 mg HQ per portion [3]. Because arbutin in pears is matrix-bound, it is conceivable that a considerable proportion of dietary arbutin may reach the distal part of the human intestinal tract, where it is a potential substrate for intestinal bacteria [2,4]. Both fecal slurries from human subjects and many individual intestinal bacteria including *Eubacterium, Enterococcus, Bacteroides*, and *Bifidobacterium* can deglycosylate arbutin to HQ [5,6]. Therefore, it is assumed that arbutin may pass into the small intestine, where it may be either absorbed or hydrolyzed by the β-glucosidase produced by the intestinal microflora, resulting in the formation of HQ. The HQ thus liberated may be absorbed by the body or undergo conjugation in either the intestine or liver [4]. Once produced, HQ has toxic and mutagenic effects *in vitro* [7].

This study aims to understand the role of metabolism by the intestinal microflora in arbutin-induced apoptosis in hepatoma HepG2 cell lines. A human fecal preparation containing intestinal microflora, known as fecalase, was used as a metabolic activation system. Initially, the cytotoxic effects of arbutin and HQ were compared. Subsequently, arbutin was incubated with fecalase to determine whether HQ could be produced by the intestinal microflora and to study the mechanism of apoptosis induced by arbutin activation through metabolism by the intestinal microflora. Apoptotic effects of fecalase-treated arbutin were investigated in HepG2 cell

---

* Corresponding authors. Address: Department of Toxicology, College of Pharmacy, Chungnam National University, 220 Gung-dong, Yuseong-Gu, Daejeon 305-764, South Korea. Fax: +82 42 825 4946 (H.G. Jeong), +82 53 810 4654 (T.C. Jeong).

E-mail addresses: taecheon@ynu.ac.kr (T.C. Jeong), hgjeong@cnu.ac.kr (H.G. Jeong).

[1] These authors contributed equally.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.08.094

cultures following filter sterilization. The data indicated that HQ may be produced by the intestinal microflora and that HQ and the pre-incubated mixture containing arbutin and its microbial metabolite HQ might be more potent inducers of cytotoxicity and apoptosis than arbutin. These findings suggest that metabolism by the intestinal microflora may be critical for activation of arbutin to its toxic metabolite HQ.

2. Materials and methods

2.1. Chemicals and materials

Reagents used in this study were purchased from the following sources: arbutin, HQ, *N*-acetyl cysteine (NAC), and 4,6-diamidino-2-phenylindole (DAPI) from Sigma Co. (St. Louis, MO, USA); the tetrazole 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corp. (Cleveland, OH, USA); DMEM, fetal bovine serum (FBS), and penicillin–streptomycin solution from Invitrogen Co. (Carlsbad, CA, USA); DeadEnd™ Fluorometric TUNEL system and caspase-3 activity assay kit from Promega (Madison, WI, USA); primers for Bcl-2 and Bax from Bioneer Co. (Daejeon, Korea); antibodies against Bcl-2, Bax, and cleaved-caspase-3 from Cell Signaling Technology (Beverly, MA, USA); and antibodies against β-actin from Santa Cruz (Santa Cruz, CA, USA). The cytotoxicity detection kit used to measure lactate dehydrogenase (LDH) release was from Roche Applied Science (Indianapolis, IN, USA). All other chemicals were of the highest commercial grade available.

2.2. Preparation of fecal suspension of human intestinal microflora

A human fecal suspension was prepared as described previously [8]. Briefly, ~30 g of feces from five human males in their twenties was collected, mixed, and suspended in 270 mL ice-cold saline. The fecal suspension was centrifuged at 100*g* for 5 min at 4 °C. The supernatant was then centrifuged at 10,000*g* for 20 min at 4 °C. The resulting precipitates (about 3 g) were resuspended in 100 ml saline and used as a source of metabolic enzymes.

2.3. Cell culture and treatment

The human hepatoma cancer cell line HepG2 was obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10% FBS, 100 μ/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 humidified incubator. Different concentrations of arbutin and HQ were added directly to cultures for cytotoxicity assays. For incubation with the human intestinal microflora suspension, arbutin at various concentrations was mixed with 1 ml of human fecal preparation (about 30 mg wet weight) and incubated at 37 °C for 20 h. Following incubation, the reaction mixture was filter-sterilized (0.2 μm pore size) and added to cultures.

2.4. Metabolism of arbutin by the human intestinal microflora

To measure the production of HQ from arbutin by a human fecal preparation, arbutin (2.5 mM) was incubated with 150 μl fecalase at 37 °C for 3 h. Methanol (400 μl) was then added to terminate the reaction, followed by centrifugation at 2500*g* for 20 min at 4 °C. Arbutin and HQ levels in supernatants were determined by HPLC. Following methanol extraction, samples (10 μl) were injected into a chromatographic system (LC-20AD; Shimadzu). The column used for separation was an Inertsil® ODS-3 (C_{18}, 4.6 × 150 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) with a guard column (4 × 3 mm, Phenomenex, USA). The HPLC mobile phase consisted of 100% methanol and 0.1% formic acid in distilled water (4:96, v/v). The analyses were performed at room temperature at a flow rate of 1 ml/min. The analyte was monitored at 288 nm.

2.5. Measurement of cell viability

HepG2 cells were plated at a density of 4×10^4 cells/500 μl in 48-well plates and after treatment with test materials or vehicle for 24 h, the MTT and LDH release assays were performed as described previously [9].

2.6. RNA preparation, semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was extracted from treated cells using the RNAiso Reagent (Takara), according to the manufacturer's instructions. Target gene expression was detected by semi-quantitative RT-PCR and real-time PCR. Bax, Bcl-2, and GAPDH were used as described previously [10,11]. Accumulated PCR products were detected directly by monitoring increases in the reporter dye (SYBR®). Product formation was monitored continuously during PCR using Sequence Detection System software (ver. 1.7; Applied Biosystems, Foster City, CA). The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of GAPDH, a housekeeping gene.

2.7. Determination of caspase-3 activity

Caspase-3 activation was determined by measuring the cleavage of a specific substrate [12]. After treatment with test materials or vehicle for 24 h, cells were lysed in a hypotonic buffer. The supernatants were collected and incubated with 100 μM of the substrate DEVD-pNA at 37 °C. The change in absorbance at 405 nm was measured using a plate reader.

2.8. Reactive oxygen species (ROS) production

ROS production in HepG2 cells was measured using the redox-sensitive fluorescent dye H_2DCFDA. After treatment with test materials or vehicle for 24 h, cells were incubated with 25 μM H_2DCFDA for 20 min. Cells were then rinsed twice with phenol red-free DMEM containing 1% FBS, and fluorescence intensities were determined using an FL600 fluorescence spectrophotometer.

2.9. Western immunoblot analysis

Following treatment with test materials, cell lysates were prepared and resolved by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were blotted onto a Hybond ECL membrane (Amersham Biosciences, Sweden) and immunoblotted with the indicated primary antibodies, as recommended by the manufacturers. Proteins were detected using the enhanced chemiluminescence detection system (Amersham Biosciences).

2.10. TUNEL assay

Apoptosis was detected by the terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay using the DeadEnd™ Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. After HepG2 cells had been treated with test materials for 24 h, chromosomal DNA was stained with DAPI and stained cells were mounted on glass slides and examined using a Carl Zeiss Axiovert 200 M microscope.