Food and Chemical Toxicology 107 (2017) 440–448

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Selective effects of whey protein concentrate on glutathione levels and apoptosis in rats with mammary tumors

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ARTICLE INFO

Article history:
Received 19 November 2016
Received in revised form
4 July 2017
Accepted 10 July 2017
Available online 11 July 2017

Keywords:
Whey protein concentrate
Glutathione
Apoptosis
7,12-dimethylbenz[*a*]anthracene
Mammary tumors

ABSTRACT

Glutathione (GSH) plays an important role in antioxidant defense and regulation of apoptosis. GSH deficiency is related to many diseases, including cancer, and increased GSH levels in cancer cells are associated with chemotherapy resistance because of resistance to apoptosis. In this study, we investigated the effects of whey protein concentrate (WPC), a precursor of GSH, in rats with mammary tumors induced by treatment with 7,12-dimethylbenz(*a*)anthracene (DMBA). DMBA treatment results in cellular changes that mimic the initiation and promotion of carcinogenesis of breast tissue. We aimed to examine the possible preventive effects of diets containing whey protein on DMBA-induced mammary tumors in rats. The results indicate that WPC (0.334 g/kg) supplementation significantly increased the liver GSH levels by 92%, and were accompanied by low Bax/Bcl-2 ratio (from 5 to 3) and cleaved caspase-3/procaspase-3 ratio (from 2.4 to 1.2) in DMBA-treated rats. Furthermore, tumor GSH levels were decreased by 47% in WPC-supplemented rats, which resulted in increased Bax/Bcl-2 ratio (from 0.9 to 2) and cleaved caspase-3/procaspase-3 ratio (from 1.1 to 2.7). In conclusion, supplementation with WPC could selectively deplete tumor GSH levels and, therefore, WPC supplementation might be a promising strategy to overcome treatment resistance in cancer therapy.

1. Introduction

Breast cancer is the most common malignancy in women worldwide and its incidence has increased globally over the last several decades. Although the overall survival rate of breast cancer increases owing to many reasons, the resistance to radiation or chemotherapy is still a main point for a successful treatment in metastasis victims. Previous studies have demonstrated that some compounds served as adjuvants in cancer treatment by decreasing intracellular levels of glutathione (GSH) in tumor cells, which made tumor cells more sensitive to radiation and chemotherapy, while restoring depressed levels of GSH in normal tissue (D'Alessio et al., 2004; Gaurav et al., 2012; Schroder et al., 1996).

GSH is a tripeptide enzymatically formed from glycine, cysteine, and glutamate, which via a two-step ATP-requiring enzymatic process. The first step is catalyzed by glutamate-cysteine ligase (GCL), which is composed of catalytic and modifier subunits (GCLC and GCLM). This step conjugates cysteine with glutamate, generating γ-glutamylcysteine. The second step is catalyzed by GSH synthase, which adds glycine to γ-glutamylcysteine to form γ-glutamylcysteinylglycine or GSH (Lu, 2013). The antioxidant reaction of GSH generates thiyl radicals (GS'), which react with oxygen to form GS-oxygen conjugates and form glutathione disulfide (GSSG) (Reed and Fariss, 1984). Nuclear factor erythroid 2-related factor 2 (Nrf2) controls several antioxidant pathways, including that of GSH production and regeneration, which is regulated by the following antioxidants: GCLC, GCLM (Gorrini et al., 2013). Changes in GSH homeostasis are associated with changes in the control of

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http://dx.doi.org/10.1016/j.fct.2017.07.024
0278-6915/

Abbreviations

(DMBA) 7,12-dimethylbenz[*a*]anthracene
(GAPDH) glyceraldehyde 3-phosphate dehydrogenase
(GSH) glutathione
(GSSG) glutathione disulfide
(GCL) glutamate–cysteine ligase
(GCLC) glutamate–cysteine ligase catalytic subunit
(GCLM) glutamate–cysteine ligase complex modifier subunit
(GR) glutathione reductase
(Nrf2) nuclear factor erythroid 2-related factor 2
(ROS) reactive oxygen species
(WPC) whey protein concentrate

apoptosis and cell cycle regulation signaling, involved in many types of disease (Forman et al., 2009; Franco et al., 2007). Apoptosis is a very tightly programmed cell death while play a critical role in maintaining the healthy balance between cell survival and cell death. Defect in apoptosis can result in cancer or autoimmunity, while excessive apoptosis may cause degenerative diseases (Hassan et al., 2014). The depletion of GSH has been shown to regulate both extrinsic and intrinsic apoptotic signaling cascades at distinct checkpoints (Franco et al., 2014).

Elevated GSH levels were observed in various types of tumors including breast, ovarian, head and neck and lung cancer (Gamcsik et al., 2012). In our previous study had revealed that the GSH levels in the blood of patients with breast cancer were lower than those in the control subjects, but were higher in tumor tissues than in the adjacent cancer-free tissue (Yeh et al., 2006). We believed that the higher GSH levels in the tumor tissue of patients with breast cancer could be associated with a higher rate of cancer cell proliferation and resistance to apoptosis and chemotherapy. However, the lower GSH levels in the blood of patients with breast cancer may have been due to the enhancement of detoxification reactions and defense against oxidative stress.

WPC is rich in cystine, a disulfide-bonded form of cysteine. Cystine is more stable than the free amino acid cysteine in the gastrointestinal tract. Therefore, WPC has been found to be an effective and safe donor of cysteine when fed to GSH-depleted animals in an immune-deficient state (Gill et al., 2000). The present study aimed to investigate the effects of WPC supplementation on GSH status and expression of apoptosis-related proteins in the blood, liver, and mammary tumor tissues of rats treated with 7,12-dimethylbenz[*a*]anthracene (DMBA) (Geyer et al., 1951). These results might provide insight into the effects of WPC on altered carcinogenic status and indicate its beneficial effects as an adjuvant in cancer treatment owing to selective effects on GSH levels and apoptosis in rats with mammary tumors.

2. Materials and methods

2.1. Materials

WPC (Immunocal, Immunotec Research Ltd., Quebec Canada) contained 90% whey protein isolate, <1.5% lactose, <0.5% fat, and <5.0% moisture, and had a solubility index of 99% at pH 4.6. The amino acid profile of WPC was provided in the Supplementary Table 1 (Micke et al., 2002). WPC was freshly prepared before each experiment and DMBA was purchased from Sigma, Saint Louis, MO, USA.

2.2. Experimental animals

Female Sprague-Dawley rats (45 d, 151–175 g) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). The rats were allowed to acclimate for 1 week prior to the study and had free access to water and standard laboratory chow. The rats were maintained in standard cages in an animal care facility and were subjected to an artificial 12:12 h light/dark cycle at 20–22 °C. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Kaohsiung Medical University (approval no. 95152).

2.3. Experimental design

The rats were randomized into four groups: Control (n = 8), Control + WPC (n = 8), DMBA (n = 12), and DMBA + WPC (n = 12). At the age of 52 d, the rats in the DMBA and DMBA + WPC groups were administered a single oral dose of DMBA (75 mg/kg). To investigate the effects of WPC, the rats in the Control + WPC and DMBA + WPC groups were supplemented with WPC (0.334 g/kg) by an intragastric tube three times a week for 18 weeks (Tsai et al., 2003). The route of intragastric administration provided a rigorously control with animal's consumption of WPC. The rats were examined weekly for tumor development and their weights recorded. At 12 h after the last treatment, rats were euthanized by cardiac puncture under Zoletil 50 anesthesia. Tumor volume was measured using calipers and calculated using the following formula (Huovinen et al., 1993): tumor volume = $\pi/6 \times$ (longest diameter) $\times$ (shortest diameter)^2.

2.4. Analysis of blood and tissue GSH status

The blood and tissue GSH and GSSG were performed by capillary electrophoresis method as previously described (Carru et al., 2002). Briefly, blood samples were collected in EDTA-containing tubes. After separating the plasma, the buffy coat was removed and the packed cells were washed three times with PBS. Aliquots of washed red blood cells (100 μL) were mixed with 5% metaphosphoric acid (MPA; 300 μL) on ice for 10 min. After centrifugation at 15,000 × *g* for 10 min at 4 °C, the supernatants were filtered through a 0.2 μm filter and diluted five times before being injected into a capillary electrophoresis system (P/ACE MDQ, Beckman Coulter, Taiwan Inc.), equipped with a fixed wavelength UV detector. Throughout all the experiments, uncoated fused-silica capillaries (75 mm I.D., 50 cm effective length) from Beckman were used. The sample was injected by the application of 0.5 psi pressure for 15 s. Before each run, the capillary was rinsed and filled with the running buffer [300 mM boric acid (pH 7.8)]. The electrophoresis performed with a constant voltage of 25 kV for 8 min at 28 °C. Beckman P/ACE MDQ software was used for instrument control. Data were quantified on the basis of corrected peak areas with migration times. To assay the GSH status in liver and tumor tissue, tissues were cut into small pieces, weighed, and homogenized with 1% MPA, then incubated on ice for 15 min. After centrifugation for 20 min at 15,000 × *g* at 4 °C, the supernatants were filtered through a 0.2 μm filter and injected into an automated capillary electrophoresis system, as indicated above. Finally, the pellets were dissolved in 1 N sodium hydroxide to measure protein concentration.

2.5. RNA extraction and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA of tumor tissues was extracted with Trizol reagent (Invitrogen, San Diego, CA, USA) and RT-PCR was conducted as previously described (Ding et al., 2008). In brief, the PCR products