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Effect of broccoli extract enriched diet on liver cholesterol oxidation in rats subjected to exhaustive exercise

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ABSTRACT

The effect of broccoli extract (BE)-enriched diet was studied in order to evaluate its ability to counteract liver cholesterol oxidation products (COPs) induced by acute strenuous exercise in rats. Thirty-two female Wistar rats were randomly divided into four groups: control diet without exercise (C), BE-enriched diet without exercise (B), control diet with acute exhaustive exercise (S) and BE-enriched diet with acute exhaustive exercise (BS). The study lasted 45 days and on the last day, rats of S and BS groups were forced to run until exhaustion on a treadmill. Glutathione-S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and cholesterol oxidation products (COPs) were determined in liver. Exhaustive exercise was clearly responsible for tissue damage, as evidenced by the increase of lactate dehydrogenase (LDH) plasma activity in the S group. Moreover, the exercise protocol reduced CAT activity in liver, while it did not affect GST, GR and GPx. BE-enriched diet raised GST, GR and CAT activities in rats of BS group. The main COPs found were 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholestanetriol, 24-hydroxycholesterol and 27-hydroxycholesterol. The BE-enriched diet led to reduced cholesterol oxidation following exhaustive exercise; the highest level of COPs was found in the S group, whereas the BS rats showed the lowest amount. This study indicates that the BE-enriched diet increases antioxidant enzyme activities and exerts an antioxidant effect towards cholesterol oxidation in rat liver, suggesting the use of phytochemicals in the prevention of oxidative damage and in the modulation of the redox environment.

1. Introduction

The interest on physical exercise has enormously risen in the last decades and a large amount of scientific literature has been published. The success of this topic partly lies on the beneficial effects induced by physical exercise on human health [1,2]. However, exhaustive exercise can be quite harmful, since it is responsible for a burst of oxidative stress, which is followed by an inflammatory response and a consequent structural damage to muscle fibers; the release of cytosolic enzymes (*i.e.* lactic dehydrogenase (LDH) and creatine kinase (CK)) in plasma, confirms the occurrence of such events [3]. Exhaustive exercise does not only affect muscle cells homeostasis and viability, but it also exerts deleterious effects on different tissues and organs. At both cardiac and hepatic levels, strenuous activity has been related to functional impairment, oxidative stress, activation of apoptotic signaling pathways and inflammation [4–6]. Due to its deleterious effects, many nutritional interventions have been studied to counteract exhaustive exercise induced damage. In this context, unfortunately, the supplementation with ROS scavengers (such as antioxidant vitamins) seems unable to prevent exhaustive exercise stress [7]. On the other hand, foods rich in indirect antioxidants or molecules able to induce antioxidant/detoxifying systems might represent an alternative nutritional approach to counteract exhaustive exercise induced stress. One example of this type of bioactive compounds is sulforaphane (SF), an isothiocyanate found

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in cruciferous vegetables (such as broccoli), which can be introduced through the diet. SF was initially studied for its promising chemopreventive activity [8], thanks to its ability to induce phase II enzymes both *in vitro* and *in vivo* [9–14]. In a recent study carried out in rats, it was also confirmed that SF treatment induces enzymes with an antioxidant/detoxifying activity (such as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione-S-transferase (GST), and glutathione reductase (GR)) in muscles, counteracting damages caused by exhaustive exercise [15].

Among biomolecules susceptible to oxidation, cholesterol is able to form a wide range of oxidation products (so-called COPs or oxysterols) in the ring and the side chain of its structure, by means of enzymatic or chemical mechanisms. They can come from exogenous (diet) and endogenous (*in vivo*) sources, and are known to be involved in fundamental functions in normal physiologic conditions, including the control of cholesterol homeostasis at cellular level [16]. The interaction with the nuclear receptor LXRα, which is highly expressed in the liver and several tissues, mediates the majority of their biological actions, thus regulating cholesterol metabolism of human body. However, there is a large research-supported evidence on the contribution of COPs to the onset and development of major chronic diseases (such as neurodegenerative processes, atherosclerosis, diabetes, osteoporosis and kidney failure), together with a series of negative biological effects (pro-inflammatory, pro-apoptotic, cytotoxic, carcinogenic and mutagenic) [17]. Moreover, it seems that COPs may also be involved on non-alcoholic fatty liver disease, thus causing liver damage [12]. Among COPs found in liver, 7α-hydroxycholesterol (7α-HC) is usually one of the most representative, as it is produced by cholesterol 7α-hydroxylase (CYP7/A1) to act as intermediate in the bile acid formation. In addition, cholesterol 27-hydroxylase (CYP27/A1), a mitochondrial P450 enzyme highly expressed in hepatocytes, is able to generate 27-hydroxycholesterol (27-HC).

To control *in vivo* oxidation processes, it would be interesting to evaluate whether exhaustive exercise is related to an increase of lipid peroxidation in liver and whether dietary vegetable extracts containing phytochemicals (such as SF and glucosinolates present in broccoli extract) could represent a strategy to prevent/counteract ROS production and lipid peroxidation, including cholesterol oxidation. Therefore, the aim of the present study was to assess the protective effect of a dietary broccoli extract (BE) against liver cholesterol oxidation in rats subjected to acute exercise, focusing on the activity of antioxidant/detoxification phase 2 enzymes.

2. Materials and methods

2.1. Materials

Bio-Rad Bradford protein assay was supplied by Bio-Rad (Hercules, CA). NADP, NADPH, NADH, FAD, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), oxidized glutathione (GSSG), reduced glutathione (GSH), 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald®), methanol, H_2O_2, EDTA, *tert*-butyl hydroperoxide, mammalian protease inhibitor mixture, cholest-5-en-3β-ol (cholesterol) (purity: 99%), β-sitosterol (purity: 60%), campesterol (purity: 37.5%), (24*S*)-ethylcholest-5,22-dien-3β-ol (stigmasterol) (purity: 95%), betulin (purity: 98%), cholest-5-en-3β,7β-diol (7β-hydroxycholesterol, 7β-HC) (purity: 90%), 5α,6α-epoxy-cholestan-3β-ol (α-epoxycholesterol, α-EC) (purity: 87%), 5β,6β-epoxy-cholestan-3β-ol (β-epoxycholesterol, β-EC) (purity: 80%), cholestan-3β,5α,6β-triol (cholestanetriol, triol) (purity: 99%) and cholest-5-en-3β-ol-7-one (7-ketocholesterol, 7-KC) (purity: 99%), were purchased from Sigma Chemical (St. Louis, MO). Tecklad 6% Mouse/Rat standard diet was supplied by Harlan Laboratories Inc. (Madison, WI). Broccoli extract capsules (Broccoli-Max® 400 mg) were purchased from Phenix srl. (Italy). *n*-hexane, chloroform and ethanol were obtained from Merck (Darmstadt, Germany). Double distilled water and the silylating agents (pyridine, hexamethyldisilazane and trimethylchlorosilane) were purchased from Carlo Erba (Milan, Italy). Potassium hydroxide and anhydrous sodium sulfate were supplied by Prolabo (Fontenay, France) and BDH (Poole, England), respectively. Cholest-5-en-3β,7α-diol (7α-hydroxycholesterol, 7α-HC) (purity: 99%) and cholest-5-en-3β,19-diol (19-hydroxycholesterol, 19-HC) (purity: 99%) were obtained from Steraloids (Newport, Rhode Island, USA). Cholest-5-en-3β,24(*S*)-diol (24(*S*)-hydroxycholesterol, 24-HC) (purity: 99%) and cholest-5-en-3β,27-diol (27-hydroxycholesterol, 27-HC) (purity: 99%) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). N°1 filters (70 mm diameter) were supplied by Whatmann (Maidstone, England). Aminopropyl solid-phase extraction (SPE) cartridges (Strata NH2–55 mm, 70A, 500 mg/3 mL) from Phenomenex (Torrence, CA, USA) were used for oxysterols purification.

2.2. Animals, diet and exercise protocol

The study and its experimental protocol were approved by the Ethics Committee of the University of Bologna (prot. N. 58897-X/10 of November 20th 2008); 32 female Wistar rats (weight: 229 ± 20 g age: 4 months) were caged in controlled conditions (22 °C and 12:12-h light-dark cycle) and supplied with water and standard chow (Harlan Laboratories Inc.) ad libitum. Data on rats' food intake were recorded for one week before the beginning of the study, as previously reported [18]; the mean food intake was 22.0 ± 1.7 g/die. Rats were randomly divided into four groups of 8 animals each: group C was fed a standard diet for 45 days and was not subjected to the exhaustive exercise protocol; group B was fed a standard diet enriched with BE for 45 days and was not subjected to the exhaustive exercise protocol; group S was fed a standard diet for 45 days and subjected to a single acute exhaustive exercise session on day 45; group BS was fed a standard diet enriched with BE for 45 days and subjected to a single acute exhaustive exercise session on day 45.

Standard diet provided macronutrients in the normal range of adequacy for rats (expressed in g/100 diet): 25 g of proteins, 36.9 g of carbohydrates, 6.2 g of lipids and suitable amounts of vitamins and minerals. BE-enriched diet was prepared by adding BE (see composition in paragraph 2.1) to the standard diet at the ratio of 0.025:1 (*w/w*; 2.5 mg of supplement/g of diet). As reported in certified product leaflet provided by the supplier, broccoli extract capsule (Broccoli-Max®) contained sulforaphane (1.2 mg, by HPLC), glucosinolates (24 mg, by HPLC), excipients (SiO_2, cellulose, Mg stearate; 45 mg) and operculum (95 mg). Considering both the data on food intake and the BE composition provided by the supplement manufacturer, BE-enriched diet provided rats with an average daily intake of 0.15 mg SF (0.55 mg SF/kg bw/day). This dose is justified by the pharmacokinetic studies carried out by Hanlon et al. [19], which demonstrated that, for a wide range of doses of orally administered SF (0.5–5 mg SF/kg bw), its bioavailability decreases with increasing dosages. Moreover, in humans, a 0.55 mg/kg bw SF dose is contained in a 100 g portion of broccoli [20], which could be easily consumed with a regular diet.

On day 38, animals were allowed to familiarize with the treadmill; they began walking on the treadmill and then they ran for 10 min at slow speed (7 m/min). On day 45, rats from S and BS groups performed an exhaustive running bout, with the treadmill slope set at +7% and speed risen up to 24 m/min. Animals were forced to run until exhaustion. When they were unable to get off the treadmill back-wall they were repositioned by hand on the treadmill front for three consecutive times before considering them exhausted [15]. Fig. 1 summarises the experimental design.