Available online at www.sciencedirect.com

SCIENCE DIRECT

Free Radical Biology & Medicine 39 (2005) 91–97

www.elsevier.com/locate/freeradbiomed

Original Contribution

β-Sitosterol modulates antioxidant enzyme response in RAW 264.7 macrophages

Marta Vivancos, Juan J. Moreno*

Department of Physiology, Faculty of Pharmacy, University of Barcelona, E-08028 Barcelona, Spain

Received 16 July 2004; revised 22 February 2005; accepted 22 February 2005
Available online 19 March 2005

Abstract

Uncontrolled production of reactive oxygen species contributes to the pathogenesis of diseases such as cancer and cardiovascular disorders. Olive oil exerts remarkable preventive effects on the development of these diseases, which may be due to the action of various components of the olive oil. In fact, several findings suggest that minor components, like phytosterols such as β-sitosterol, are responsible, at least in part, for these beneficial effects. Our results show that β-sitosterol reverts the impairment of the glutathione/oxidized glutathione ratio induced by phorbol esters in RAW 264.7 macrophage cultures. These data can be correlated with the increase in manganese superoxide dismutase and glutathione peroxidase activities and the decrease in catalase activity. We also demonstrate that the effects of β-sitosterol on antioxidant enzymes depend on the estrogen/phosphatidylinositol 3-kinase pathway.

Keywords: Phytoestrogens; Glutathione; Superoxide dismutase; Glutathione peroxidase; Catalase; Free radicals

Phytosterols or plant sterols are the plant counterparts of cholesterol in animals. They are present in the diet in several forms, but the two most abundant are β-sitosterol and campesterol [1]. In vitro and in vivo studies suggest that phytosterols offer protection from the most common cancers in developed countries. Their dietary consumption is poorer in developed countries (80 mg/day) than in Asian countries (400 mg/day), where the incidence and mortality for these cancers are lower [2]. However, the exact mechanism by which dietary phytosterols offer this protection is not fully understood.

The Mediterranean diet is healthy and has many protective effects against cancer and heart disease development [3,4], and olive oil is one of its representative components. Epidemiological studies indicate that olive oil strongly prevents the development of these diseases [5,6]. The uncontrolled production of reactive oxygen species (ROS) contributes to the pathogenesis of cardiovascular disease [7,8] and cancer [9], and mononuclear cells like macrophages that infiltrate the atheroma plaque or tumor are a major source of ROS [8,9]. Recent observations have revealed that stimulated macrophages from rats fed an olive oil diet release less superoxide anion ($O_2^{\bullet -}$) than cells from animals fed a fish oil or corn oil diet [10]. Although this may be ascribed to various components, several findings suggest that minor components like β-sitosterol are responsible, at least in part, for these beneficial effects. Furthermore, β-sitosterol (50 μM) does not scavenge $O_2^{\bullet -}$ but decreases $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) levels in phorbol ester-stimulated macrophages [11].

Because macrophages present membrane estrogen receptors [12], β-sitosterol shows binding affinity for these estrogen receptors [13], and estrogens act as antioxidants, at least to some extent, via the stimulation

Abbreviations: ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide anion; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; GSH, glutathione; GSSG, glutathione oxidized; GPx, glutathione peroxidase; SOD, superoxide dismutase; NBT, *p*-nitroblue tetrazolium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PI3-kinase, phosphatidylinositol 3-kinase.

* Corresponding author. Fax: +34 93 403 5901.
E-mail address: jjmoreno@ub.edu (J.J. Moreno).

0891-5849/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2005.02.025

of antioxidant enzymes [14,15], we aimed to determine the effect of β-sitosterol on antioxidant enzymes. To address this question, we measured β-sitosterol-induced changes in glutathione content as well as in superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) activities. The identification of a regulatory pathway through which β-sitosterol may reduce cellular ROS levels through the modulation of antioxidant enzymes would provide a better understanding of the beneficial effects of nutrients which, like olive oil, contain this phytosterol as a minor component.

Materials and methods

Materials

Phorbol 12-myristate 13-acetate (PMA), β-sitosterol, phenylmethylsulfonyl fluoride (PMSF), aprotinin, NADPH, glutathione (GSH), glutathione oxidized (GSSG), 5,5′-dithiobis(2-nitrobenzoic acid), GPx from bovine erythrocytes, glutathione reductase from *Saccharomyces cerevisiae,* SOD from bovine erythrocytes, catalase from human erythrocytes, *p*-nitroblue tetrazolium (NBT), tamoxifen, wortmannin, cycloheximide, and cumene hydroperoxide were from Sigma Chemical Co. (St. Louis, MO, USA). ICI 182,780 was from Tocris (Bristol, UK). Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin G, and streptomycin were from BioWhittaker Europe (Verviers, Belgium). All other reagents were of analytical grade. β-Sitosterol, tamoxifen, wortmannin, and cycloheximide were dissolved in dimethyl sulfoxide and diluted in medium to keep the final concentration of dimethyl sulfoxide below 0.1%. Control cells were incubated with the vehicle.

Culture of RAW 264.7 macrophages

Murine RAW 264.7 macrophages (TIB-71) from American Type Culture Collection (Manassas, VA, USA) were grown in DMEM containing 10% FCS, penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}), in a 95% air–5% CO_2 humidified atmosphere at 37°C. Cells were scraped off and passed to tissue culture 100- or 60-mm dishes (Costar, Cambridge, MA, USA) for experimental purposes. Ethidium bromide/acridine orange staining was used to assess cell viability.

Estimation of cellular GSH and GSSG

Total glutathione (GSH plus GSSG) and GSSG were measured in a recycling assay using 5,5′-dithiobis(2-nitronenzoic acid) and glutathione reductase. Briefly, macrophages were lysed and deproteinized in 3% 5-sulfosalicylic acid. Whole-cell lysates were cleared at 4°C by centrifugation at 20,000*g* and the supernatant was used to measure total and oxidized glutathione. Total glutathione was read from a GSH standard curve, prepared in 5-sulfosalicylic acid. For the GSSG assay, 100 μl of supernatant was incubated with 2 μl of 2-vinylpyridine and 6 μl of triethanolamine for 60 min on ice. GSSG standards were treated in the same way, and the GSSG content of the samples was calculated from a GSSG standard curve. Reduced GSH was calculated by subtracting GSSG from total glutathione.

Antioxidant enzyme assays

Cells were scraped off and harvested by centrifugation. Then the cell pellets were resuspended in 1 ml lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.02% NaN_3; 100 μg ml^{-1} PMSF; 1 μg ml^{-1} aprotinin; 1% Triton X-100). After 30 min at 4°C, enzyme assays were carried out without further delay.

SOD activity was determined by the modified NBT method described by Spitz and Oberly [16], an indirect assay based on the competition reaction between SOD and NBT. The rate of increase at 560 nm for 5 min indicates the reduction of NBT by superoxide anion. Varying amounts of total protein were added to the reaction until maximal inhibition was detected by spectrophotometry. SOD activity was determined as the amount of protein needed to achieve half-maximal inhibition of the NBT reaction. Mn SOD activity was quantified in the presence of 5 mM NaCN, which inhibits only Cu-Zn SOD. SOD values were normalized to protein content (μU SOD/μg protein). Total protein was measured by the Bradford method [17] using the Bio-Rad protein assay, with bovine serum albumin as standard.

GPx activity was measured by the cumene hydroperoxide/GSSG recycling method [18]. Samples (150 μl) were added to wells in a 96-well plate. Fifty microliters of the GSSG recycling mix (10 mM GSH, 1 mM NADPH, 4 U/glutathione reductase in 0.5 M H_2NaPO_3, pH 7.8, 37°C) was added to each sample, including a blank containing only sample buffer, and allowed to warm at 37°C for 2–3 min. Fifty microliters of 10 mM cumene hydroperoxide was added and the oxidation of NADPH was monitored at 340 nm every 15 s for 15 min at 37°C. The most linear portion of the slope was determined and used for all samples and blanks. After subtracting spontaneous NADPH oxidation (blank), glutathione peroxidase activity was expressed as μU/μg protein.

Catalase activity was measured by monitoring the decrease in absorption of H_2O_2 at 240 nm [19]. Briefly, 100 μl of the cell lysate was added to a 0.5-ml quartz cuvette containing 400 μl of 20 mM H_2O_2 in PBS (25°C) and mixed thoroughly by pipetting. Absorbance was immediately monitored at 240 nm every 2 s for 2 min and the most linear portion of the curve (10–40 s) was used. Catalase activity was expressed as μU/μg protein.