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SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 2003–2008
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Antioxidant effect of hydroxytyrosol, a polyphenol from olive oil: scavenging of hydrogen peroxide but not superoxide anion produced by human neutrophils

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Received 10 March 2004; accepted 25 June 2004

Abstract

Hydroxytyrosol (HT) (also known as dihydroxyphenylethanol (DPE)) is a polyphenol extracted from virgin olive oil. HT is known to exert an antioxidant effect but the mechanism of action and the identity of the reactive oxygen molecule(s) targeted are not known. In this study, we show that HT inhibits luminol-amplified chemiluminescence of human neutrophils stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol myristate acetate (PMA) and opsonized zymosan. This effect was dose-dependent and occurred immediately after the addition of HT. However, HT had no effect on lucigenin-amplified chemiluminescence, suggesting that it does not inhibit NADPH oxidase activation or scavenge superoxide anions. Furthermore, HT inhibited H_2O_2-dependent-dichlorofuoroscein (DCFH) fluorescence of activated neutrophils, as measured by flow cytometry. Finally, HT inhibited luminol-amplified chemiluminescence in a cell-free system consisting of H_2O_2 and HRPO. These results suggest that HT, a polyphenol derived from olive oil, could exert its antioxidant effect by scavenging hydrogen peroxide but not superoxide anion released during the respiratory burst.

Keywords: Hydroxytyrosol; H_2O_2; Neutrophils; Respiratory burst; Antioxidant

1. Introduction

Reactive oxygen species (ROS) play key role in many physiological and pathogenic processes, including signal transduction, inflammation, aging, neurodegeneration and atherosclerosis [1]. H_2O_2 is a ubiquitous ROS produced by many cell types. It can activate signaling processes and induce cytotoxicity in many cells [2]. There is currently intense pharmacological research to find agents that target specific ROS molecules such as H_2O_2. Epidemiologic studies support the beneficial effects of the Mediterranean diet on human health, particularly in the prevention of cardiovascular diseases and some cancers [3]. This diet is characterized by high intake of fruits, vegetables and olive oil, which provide large amounts of antioxidants and vitamins. Virgin olive oil is rich in phenolic products, which have been reported to be strong free radical scavengers [4]. Hydroxytyrosol (HT) (also known as dihydroxyphenylethanol (DPE)), the most active olive polyphenol, has antithrombotic activities such as inhibition of LDL oxidation [5], platelet aggregation [6], and endothelial cell activation [7]. HT is known to exert an antioxidant effect, but its mechanism of action is not clear. Visioli et al. [4] and Leger et al. [8] have suggested that HT and olive oil waste water are potent scavengers of superoxide anions.

The phagocyte NADPH oxidase [9,10] and endothelial NADPH oxidase [11] are sources of ROS in humans. The phagocyte respiratory burst is a coordinated series of metabolic events whereby activated phagocytes, such as monocytes, macrophages and neutrophils, reduce molecular oxygen to a variety of toxic reactive oxygen species [12]. The initial product of the respiratory burst is

Abbreviations: HT, hydroxytyrosol; DPE, dihydroxyphenylethanol; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; ROS, Reactive oxygen species; DCFH-DA, 2′,7′-dichlorofuoroscin-diacetate; HRPO, horseradish peroxidase; phox, phagocyte oxidase; SOD, superoxide dismutase

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doi:10.1016/j.bcp.2004.06.023

superoxide anion ($O_2^{\bullet -}$), and subsequent reactions lead to the formation of other toxic agents including H_2O_2, HOCl and possibly the hydroxyl radical ($OH^{\bullet}$) [12,13]. The enzyme catalyzing the one-electron reduction of oxygen is known as NADPH oxidase [9,10], a multicomponent enzyme which is dormant in resting cells, the components of which are divided between the cytosol and the membrane. Following neutrophil activation, the cytosolic components translocate to the membrane, where they join with the membrane-bound components to form a fully functional oxidase [14,15]. Macrophage and neutrophil NADPH oxidase can have prooxidant effects when ROS are produced in the extracellular medium. They are involved in tissue injury, causing inflammatory diseases [12] such as rheumatoid arthritis, inflammation and ischemia-reperfusion injury.

HT is shown to exert an antioxidant effect but its molecular target is not clear. In this study we show that HT reacts with H_2O_2 rather than O_2^- released during the neutrophil respiratory burst.

2. Materials and methods

2.1. Materials

Luminol, isoluminol, lucigenin, cytochrome *c*, fMLP, PMA, zymosan, BSA, superoxide dismutase (SOD), catalase and HRPO were from Sigma. Ficoll and Dextran T500 were from Pharmacia. HBSS, HEPES and glucose were from Gibco. HT was from Cayman. Human recombinant TNF (2×10^5 U/ml) was from Genzyme. 2′,7′-dichlorofuoroscin-diacetate (DCFH-DA) was from Acros Fine Chemicals. Stock solutions of DCFH-DA (50 mmol/l) and FMLP (10^{-2} mol/l) were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. The different solutions were diluted in phosphate-buffered saline (PBS) immediately before use.

2.2. Isolation of human neutrophils

Venous blood was collected from healthy adult volunteers and neutrophils were isolated by dextran sedimentation and density gradient centrifugation as previously described [15,16]. Erythrocytes were removed by hypotonic lysis. Following isolation, the cells were resuspended in appropriate medium, such as Hank's balanced salt solution (HBSS). A cell count was performed and cell viability was determined using the trypan blue exclusion method.

2.3. Neutrophil chemiluminescence

Following isolation, cells were resuspended in HBSS at a concentration of 5 million per ml. Cell suspensions (5×10^5) in 0.5 ml HBSS containing 10 μM luminol or 50 μM lucigenin in the presence or absence of HT were preheated to 37 °C in the thermostated chamber of the luminometer (Berthold–Biolumat LB937) and allowed to stabilize. After a baseline reading was established, cells were stimulated with 10^{-6} M fMLP, 100 ng/ml PMA or 0.5 mg/ml opsonized zymosan. Changes in chemiluminescence were measured over a 30 min period. We also used the technique described by Lundqvist and Dahlgren [17] to measure extracellular ROS production. The above conditions were used, except that 10 μM isoluminol and 5 U HRPO were used instead of luminol.

2.4. Cell-free H_2O_2-chemiluminescence assay

Stimulated neutrophils were replaced by H_2O_2. The reaction mix contained 80 μM H_2O_2, 5 U HRPO and 10 μM luminol in PBS, with or without HT. Changes in chemiluminescence were measured over a 10 min period.

2.5. Flow cytometry

Intracellular H_2O_2 production was measured using a flow cytometric assay derived from the technique described by Bass and co-workers [18,19]. Whole blood samples were preincubated in 2′,7′-DCFH-DA (100 μmol/l) in a water bath with gentle horizontal agitation at 37 °C in presence or absence of HT (13 μmol). Samples were then treated with TNF (100 U/ml) or PBS for 30 min and then with fMLP (10^{-6} M) or PBS for 5 min DCFH-DA diffuses into cells and is hydrolyzed into 2′,7′-dichlorofluoroscein (DCFH). During the PMN oxidative burst, non fluorescent intracellular DCFH is oxidized to highly fluorescent DCF by H_2O_2 in the presence of peroxidase. Red blood cells in whole blood were lysed using FACS lysing solution (Becton Dickinson). After one wash ($400 \times g$ for 5 min), white blood cells were resuspended in 1% paraformaldehyde-PBS. Flow cytometric analysis was performed with a Becton Dickinson FACScalibur (Immunocytometry Systems) equipped with a 15 mW, 488 nm argon laser. The data were analyzed using Cell Quest software (Becton Dikinson) and the mean fluorescence intensity (MFI) was used to quantitate the responses. The effect of agonists on H_2O_2 production was calculated by using a stimulation index (SI), namely the ratio of MFI-stimulated cells to that of unstimulated cells.

3. Results

3.1. Hydroxytyrosol (HT) inhibits luminol-amplified chemiluminescence of human neutrophils, independently of the stimulus

To analyze the effect of HT on the human neutrophil respiratory burst, neutrophils were incubated in the presence or absence of 10 μM HT for 15 min and chemiluminescence was measured using luminol as probe.