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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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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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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.

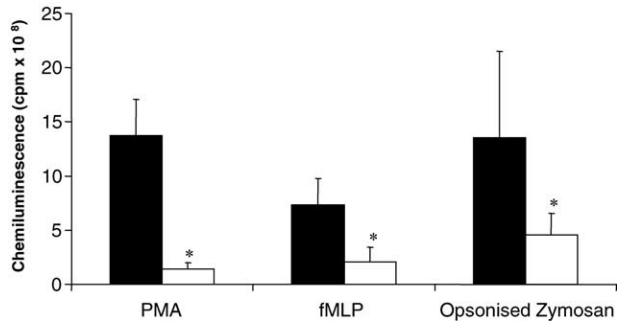

Fig. 1. Effect of hydroxytyrosol (HT) on luminol-amplified chemiluminescence of human neutrophils. Human neutrophils (5×10^5) were incubated in the presence or absence of HT (10 μM), and stimulated with fMLP (10^{-6} M), PMA (100 ng/ml) or opsonized zymosan (0.5 mg/ml). Luminol-amplified chemiluminescence was measured during 30 min (mean ± S.E.M. of five experiments, * $P < 0.05$).

Fig. 1 shows that HT inhibits luminol-amplified chemiluminescence of neutrophils stimulated with the chemotactic peptide fMLP, the protein kinase C activator PMA, and opsonized zymosan particles. The effect of HT was dose-dependent on fMLP-, PMA- (Fig. 2) and zymosan-activated neutrophils (data not shown), with an inhibitory effect starting at a concentration as low as 1 μM. However, a kinetic study (Fig. 3) showed that this effect was not time-dependent, as it occurred immediately after the addition of HT. PMA-induced chemiluminescence was inhibited by SOD (150 U) and by catalase (150 U) suggesting that this reaction depends on O_2^- and H_2O_2. Reports by Halliwell [20,21] showed that polyphenol compounds can generate H_2O_2. To check if HT generates high toxic doses of H_2O_2, we first checked that HT alone did not produce a chemiluminescence reaction in the presence of luminol

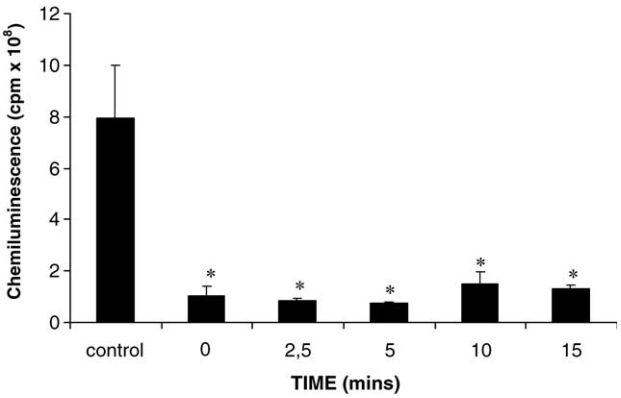

Fig. 3. Kinetics of the effect of HT on luminol-amplified chemiluminescence of human neutrophils. Human neutrophils (5×10^5) were incubated in the presence or absence of HT (10 μM) for the indicated times and then stimulated with PMA (100 ng/ml). Luminol-amplified chemiluminescence was measured for 30 min (mean ± S.E.M. of three experiments, * $P < 0.05$).

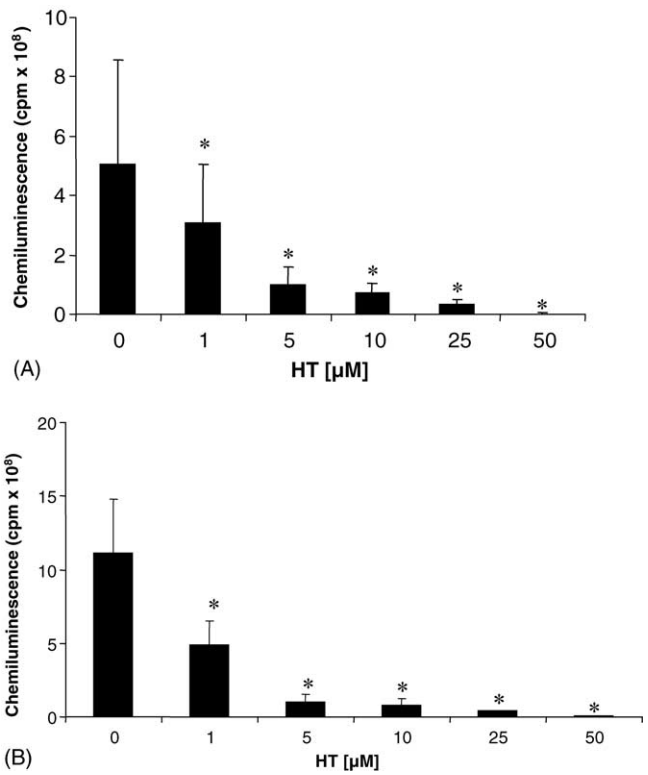

Fig. 2. Dose-dependent effect of HT on FMLP and PMA activated neutrophils. Human neutrophils (5×10^5) were incubated in the presence or absence of HT (10–100 μM), then stimulated with fMLP (10^{-6} M) (**A**); or PMA (100 ng/ml) (B). Luminol-amplified chemiluminescence was measured during 30 min (mean ± S.E.M. of three experiments, * $P < 0.05$).

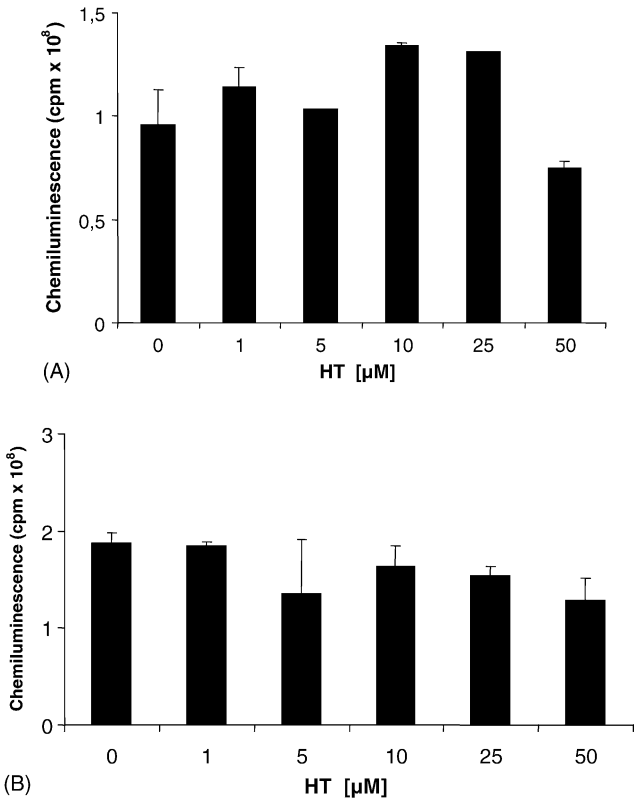

Fig. 4. Effect of HT on lucigenin-amplified chemiluminescence of human neutrophils. Human neutrophils (5×10^5) were incubated in the presence or absence of HT (10 μM), then stimulated with fMLP (10^{-6} M) (A), PMA (100 ng/ml) (B). Lucigenin-amplified chemiluminescence was measured for 30 min.

and HRPO (data not shown). We also pretreated HT with catalase, and found that the inhibitory effect of HT was not reversed (data not shown). As fMLP, PMA and opsonized zymosan activate NADPH oxidase via different transduction pathways, these results suggest that HT does not affect a specific transduction pathway, but rather appears to directly inhibit a final common biochemical target such as the NADPH oxidase enzyme or by scavenging reactive oxygen species.

3.2. Hydroxytyrosol (HT) has no effect on lucigenin-amplified chemiluminescence of human neutrophils

To investigate the effect of HT on superoxide anion production, we first used the cytochrome *c* reduction assay, a specific method of measuring superoxide anions. We found that HT directly reduced cytochrome *c*, interfering with the assay (data not shown). It is believed that lucigenin-amplified chemiluminescence of human neutrophils reflects extracellular superoxide anions, and we therefore, used this technique to analyze the effect of HT on neutrophil superoxide production. The results show (Fig. 4) that HT has no effect on lucigenin-amplified chemiluminescence of human neutrophils stimulated with fMLP or PMA. It is noteworthy that SOD inhibited lucigenin-amplified chemiluminescence but catalase like HT has no effect on this reaction. Also, HT had no effect on lucigenin-amplified chemiluminescence when xanthine/xanthine oxidase was used to produce superoxide anions; however, luminol- and HRPO-amplified chemiluminescence was inhibited (data not shown). These results suggest that HT does not affect NADPH oxidase activity or scavenge superoxide anions.

3.3. Hydroxytyrosol (HT) inhibits DCFH-detected H_2O_2 production as measured by flow cytometry

To confirm the results obtained above, suggesting that HT reacts directly with H_2O_2, we used a different technique to measure H_2O_2 production by intact neutrophils. The cell-permeant, oxidation-sensitive dye DCFH is non fluorescent until oxidized by H_2O_2. Fig. 5(A) shows that stimulation of DCFH-loaded neutrophils with TNF and fMLP led to an increase in DCFH oxidation detected by flow cytometry analysis. However, in the presence of HT, the increase in DCF fluorescence was abrogated, suggesting that HT reacts with intracellular H_2O_2. Because this techniques detects intracellular H_2O_2, SOD and catalase had no effect. Fig. 5(B) summarizes the results obtained in three different experiments using a stimulation index (SI), namely the ratio of MFI-stimulated cells to that of unstimulated cells. TNF alone and fMLP alone induced a moderate increase in fluorescence. The combination of TNF and fMLP produced a clear increase in H_2O_2. This effect was completely inhibited in HT-treated samples.

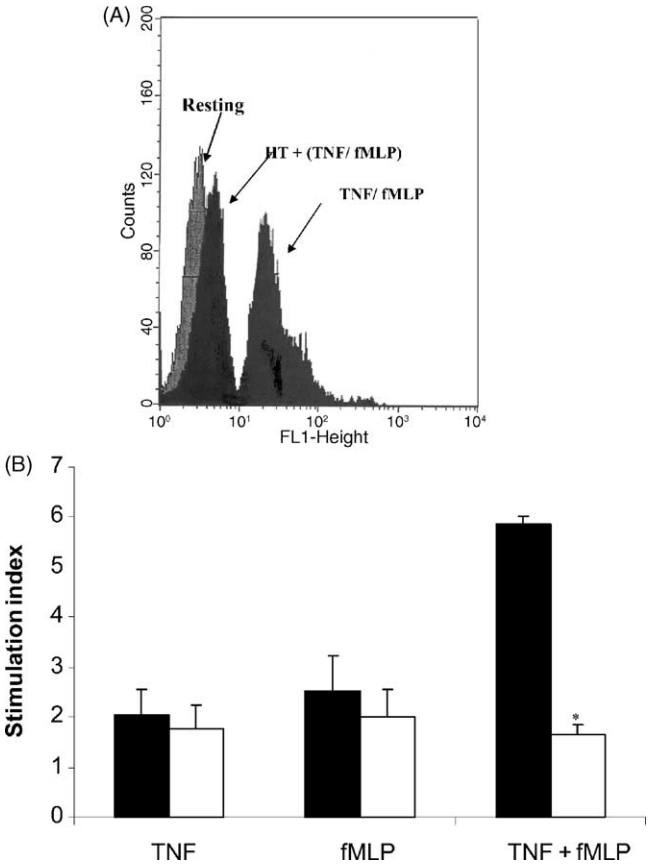

Fig. 5. Effect of HT on DCFH-detected H_2O_2 production, as measured by flow cytometry. Whole blood samples were preincubated in 2′,7′-DCFH-DA (100 μmol/l) at 37 °C in the presence or absence of HT (13 μmol). Flow cytometric analysis was performed with a Becton Dickinson FACScalibur. (A) Representative FACS profile of neutrophil stimulation and the effect of HT (Counts: number of neutrophils; FL1-Height: DCF fluorescence intensity). (B) Results from three different experiments: The mean fluorescence intensity was used to quantitate the responses. The effect of agonists was calculated using a stimuation index (SI), namely the ratio of MFI of stimulated cells to that of unstimulated cells. (mean ± S.E.M. of three experiments, * $P < 0.05$).

3.4. Hydroxytyrosol (HT) inhibited luminol-amplified chemiluminescence in a cell-free system consisting of H_2O_2 and HRPO

To investigate whether HT interferes directly with H_2O_2, we tested the effect of HT directly in a cell-free system consisting of H_2O_2, luminol and HRPO. The results (Fig. 6) showed that HT strongly inhibited chemiluminescence generated by this system. These results strongly suggest that HT reacts with H_2O_2.

4. Discussion

Hydroxytyrosol (HT) is known to have antioxidant effects but its mechanism of action is not known. In this study, we show that HT inhibits the luminol-amplified chemiluminescence of neutrophils stimulated with fMLP,

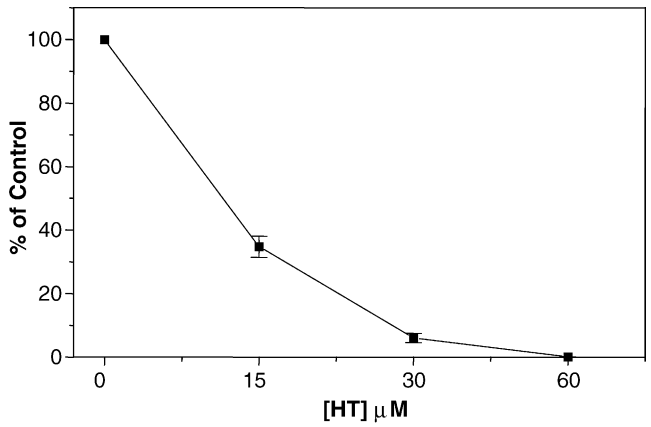

Fig. 6. Effect of HT on H_2O_2-HRPO cell-free chemiluminescence. H_2O_2 (80 μM) and luminol (10 μM) were incubated in 0.5 ml PBS in the presence or absence of HT. After 2 min, 5 U HRPO was added and changes in chemiluminescence were measured for 10 min. (mean ± S.E.M. of four experiments).

PMA and opsonized zymosan. The inhibitory effect occurs immediately after the addition of HT. However, HT has no effect on lucigenin-amplified chemiluminescence. Furthermore, HT also inhibited the H_2O_2-dependent DCF fluorescence of activated neutrophils, as measured by flow cytometry. HT has a clear inhibitory effect on the chemiluminescence of H_2O_2/HRPO cell-free system. These results suggest that HT exerts its antioxidant effect by scavenging hydrogen peroxide but not superoxide anion.

HT inhibits the luminol-amplified chemiluminescence of human neutrophils stimulated with the chemotactic peptide fMLP, the protein kinase C activator PMA, and opsonized zymosan particles. A kinetic study showed that this effect was not time-dependent, because it occurred immediately after the addition of HT. As fMLP, PMA and opsonized zymosan activate NADPH oxidase via different transduction pathways, these results suggest that HT does not affect a specific transductional pathway, but rather directly inhibits a final common biochemical target like the NADPH oxidase enzyme or scavenges reactive oxygen species. In a first attempt to investigate the effect of HT on superoxide production, we used the cytochrome *c* reduction assay, a specific technique to measure superoxide. We found that HT directly reduced cytochrome *c*, interfering with the assay, and hindering the interpretation of the data (not shown). This effect of HT on cytochrome *c* was also observed by De la Puerta et al. [23]. It is believed that lucigenin-amplified chemiluminescence of human neutrophils reflects extracellular superoxide anions [22,24]. We thus used this technique to analyze the effect of HT on neutrophil superoxide production. We found that HT had no effect on lucigenin-amplified chemiluminescence of human neutrophils stimulated with fMLP or PMA. This suggested that HT does not affect NADPH oxidase activity or scavenge superoxide anions.

The luminol-amplified chemiluminescence of human neutrophils can be used to determine both extracellularly released and intracellularly retained reactive oxygen species, because luminol is a membrane-permeable molecule. It is established that luminol-amplified chemiluminescence is dependent on H_2O_2 and peroxidases such as cytosolic peroxidases and myeloperoxidase [24,25]. We also found that HT inhibits isoluminol-HRPO-amplified chemiluminescence of human neutrophils, showing that it reacts with an extracellular component of the reaction such as H_2O_2 or HRPO, (data not shown) and only addition of H_2O_2 reversed the inhibitory effect of HT. HT also inhibited H_2O_2/HRPO cell-free chemiluminescence. These results strongly suggested that HT reacts with H_2O_2. To confirm this result we used a more specific technique to measure H_2O_2 production by intact neutrophils, based on DCFH oxidation by H_2O_2. The results showed that HT completely abrogated this reaction, suggesting that it reacts with intracellular H_2O_2.

The beneficial effects of the Mediterranean diet on human health, particularly in the prevention of cardiovascular diseases and some cancers, are widely recognized [3]. This diet is characterized by a high intake of virgin olive oil, which is rich in phenolic products which have been reported to exert a strong free radical scavenging action [3]. HT, the most active olive polyphenol, has antithrombotic activities such as inhibition of LDL oxidation [5], inhibition of platelet aggregation [6], and inhibition of endothelial expression of tissue factor [7]. Recent data show that olive polyphenols are cell-permeable and dose-dependently absorbed, and that at very low doses they exert antioxidant activities in vivo [26–28]. They increase plasma antioxidant capacity and reduce isoprostane release, both in humans [29] and in sidestream-smoke-exposed rats [30]. These effects of HT could be mediated by its H_2O_2 scavenging properties described in this study.

Because H_2O_2 is an ubiquitous ROS produced by many cell types [31], and can activate signaling processes and induce cytotoxicity in many cells at the origin of numerous health disorders, there is intense ongoing research to find pharmacological agents which target a specific ROS molecule such as H_2O_2. HT could provide a useful pharmacological tool to analyze the role of hydrogen peroxide in many biological and pathological processes.

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