Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H$_2$O$_2$ induced lipid peroxidation in renal tubular epithelial cells

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1. Introduction

Reactive oxygen species play a key role in the pathogenesis of renal injury. The abundance of polyunsaturated fatty acids makes the kidney an organ particularly vulnerable to ROS attack (Kubo et al., 1997): lipid peroxidation has been regarded as a key step common to the mechanism of a wide range of renal diseases, as tubulointerstitial alterations (Martin-Mateo et al., 1999). The generation of H$_2$O$_2$ has been implicated in the pathogenesis of several forms of acute tubular cell injury, where the lipid peroxidation process plays a central role (Salahudeen, 1995). It has been shown that severity of H$_2$O$_2$ induced cell death is directly related to the cell membrane unsaturated fatty acids (UFA) content and to the degree of lipid peroxidation (Sheridan et al., 1996). Dietary intake of foods rich in antioxidants, as phenolic compounds, is associated with reduced risk of renal dysfunction in epidemiological studies (Singh et al., 2006). There is a continuing interest to define the preventive effects of phenols against reactive oxygen species mediated degenerative diseases. Phenolic compounds are important bioactive biomolecules that are of increasingly interest for their ability to exert modulatory actions in cells by interacting with a wide spectrum of molecular targets central to the cell signaling machinery. Their potential antioxidant functions are also widely discussed from the standpoint of inhibition of lipid peroxidation (Salah et al., 1995), by virtue of potential of the free radical scavenging and metal chelating properties (Kandaswami and Middleton, 1994; Rice-Evans et al., 1996; Soobrattee et al., 2005). Extra virgin olive oil phenolic compounds have been shown to be strong antioxidant, protecting lipids from oxidation: hydroxytyrosol (HT), 3,4-dihydroxyphenylethanol (Fig. 1), and its precursors are the most active, due to their catecholic moiety (Montedoro et al., 1992a,b) and exhibit a series of in vitro biological activity. At nutritional relevant concentration HT possesses a marked antioxidant activity, scavenging peroxyl (Saija et al., 1998), other free radicals (Gordon et al., 2001) and reactive nitrogen species (de la Puerta et al., 2001; Deiana et al., 1999), or breaking peroxidative chain reactions and preventing metal ion catalyzed production of reactive oxygen species (Gutierrez et al., 2001). HT also prevents LDL oxidation in vitro and ex vivo (de la Torre-Carbot et al., 2007, and references therein). In addition there are some data demonstrating antioxidant effect on cultured cells: HT counteracts cytotoxicity of reactive oxygen species to epithelial CaCo-2 cells (Manna et al., 1997), melanoma cells (D’Angelo et al., 2005) and H$_2$O$_2$ induced oxidative stress in intact human erythrocytes (Manna et al., 1999). Its antioxidant action has been demonstrated also in vivo: HT treatment decreases isoprostane excretion both in rats (Visioli et al., 2000c) and humans (Visioli et al., 2000a). Several animal and human studies, as reviewed by de la Torre-Carbot et al. (de la Torre-Carbot et al., 2007), have shown that HT is
bioavailable. Once absorbed, it undergoes extremely extensive first-pass intestinal/hepatic metabolism in the body (Bonanome et al., 2000; D’Angelo et al., 2001; de la Torre-Carbot et al., 2007; Miro-Casas et al., 2003a,b; Vissers et al., 2002) thus its biological activity is more likely to be linked to its metabolites rather than to the compound itself. In vivo glucuronide, sulfate and methyl conjugates of HT in plasma and urine have been described (D’Angelo et al., 2001; de la Torre-Carbot et al., 2007; Del Boccio et al., 2003; Manna et al., 2000; Miro-Casas et al., 2003a,b; Tan et al., 2003; Tuck et al., 2001, 2002; Tuck and Hayball, 2002; Vissers et al., 2000b; Vissers et al., 2002); the major identified metabolite in humans is the methylated derivative homovanillic alcohol (HVA). 4-hydroxy-3-methoxy-phenylethanol, (Fig. 1), product of COMT activity, that is also naturally present in extravirgin olive oil (Manna et al., 2000; Tuck et al., 2002). A significant linear regression was found between the administered HT and the HVA detected in the urine of volunteers (Caruso et al., 2001); in the kidney of rats treated with labeled HT, HVA was present as the major metabolite (D’Angelo et al., 2001). In vitro studies suggested that HT could be a preferential substrate, with respect to the endogenous substrates as dopamine, for COMT in vivo (Manna et al., 2000), thus increasing the basal level of HVA. HVA has been reported to exert an antioxidant activity comparable to that of HT in simple chemical systems (Turner et al., 2005). As HT is absorbed, metabolized and renal excretion represents its preferential disposition (D’Angelo et al., 2001), it is likely that both HT and its metabolites, as HVA, may exert a biological effect in the renal compartment. The aim of our study was to compare the antioxidant activity of HT and its metabolite HVA in the same cell culture system, investigating the capacity of the two phenols to inhibit H₂O₂ induced oxidative damage in LLC-PK1 cells, a porcine kidney epithelial cell line that retains characteristics of the proximal tubular epithelium (Perantoni and Berman, 1979), and therefore they have been used for studying the tubular transport of various solutes (Schlatter et al., 2006) and the mechanisms of kidney toxicity (Baek et al., 2006; Cohly et al., 1998). The study of the antioxidant activity of a compound on cultured cells provides relevant information to elucidate the mechanism underlying the antioxidant effect and the structure-activity relationship. Oxidative cell injury due to H₂O₂ exposure was evaluated as MDA production and through more sensible and precise markers of the lipid peroxidation process, that plays a central role in mediating early cytotoxicity following H₂O₂ oxidant injury (Salahudeen, 1995): the modification of the profile of the major oxidizable membrane lipids, UFA and cholesterol, and the membrane antioxidant α-tocopherol.

2. Materials and methods

2.1. Materials

All solvents used were HPLC grade (Merck, Darmstadt, Germany). Fatty acids standards, cholesterol, 5-cholen-3β-ol-7-one (7-keto), α-tocopherol, H₂O₂, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and HVA were purchased from Sigma Chemical (St. Louis, MO). Desferal (defereroxamine methanesulfonate) was purchased from CIBA-Geigy (Basel, Switzerland). HT was purchased from Cayman Chemical Company (Ann Arbor, MI). Cell culture materials were purchased from Invitrogen (Milano, Italy). All other reagents and chemicals were of analytical grade.

2.2. Cell culture

LLC-PK1 cells (a porcine renal epithelial cell line with proximal tubule epithelial characteristics) were obtained from the European Collection of Animal Cell Cultures, ECACC (Salisbury, UK) and grown in Medium 199, containing 10% foetal calf serum and penicillin (100 U/mL) streptomycin (100 μg/mL), at 37 °C, under a humidified atmosphere of 5% CO₂. For experimental studies cells were plated at a density of about 5 × 10⁴/mL and grown until reaching sub-confluence in Petri dishes.

2.3. Cytotoxic activity

The cytotoxic effect caused by exposure for 1 h to increasing concentration of H₂O₂ (10–250 μM in water solution) and 24 h to HT and HVA (25–2500 μM in water solution) was assessed by the MTT colorimetric assay (Mosmann, 1983).

2.4. Determination of HVA as HT metabolite

In order to determine HT metabolism to HVA in LLC-PK1, cells at the sub-confluence (10 × 10⁴/Petri dishes) were incubated at 37 °C for different lengths of time (5 min–4 h) with HT 250 μM in 10 mL of PBS. Following exposure, the medium was removed and cells were washed with ice-cold PBS and rapidly lysed on ice using aqueous MeOH (50/50, v/v) containing HCl (0.1%). Lysed cells were scraped and left on ice for 45 min, and then centrifuged at 500g for 5 min at 4 °C to remove unbroken cell debris and nuclei. The supernatants were recovered and analysed, together with the medium, by an Agilent Technologies (Palo Alto, CA) 1100 liquid chromatograph equipped with a diode array detector, HPLC-DAD. HT and HVA were detected at 280 nm, using a Varian column, Inertsil 5 C-8 250 × 4.6 mm, with a mobile phase of MeOH/CH₃CN (50/50, v/v) (A) and H₂O/H₃PO₄ (99/1, v/v) (B) at a flow rate of 1.5 mL/min. The protein concentration in the supernatants was determined by the Bradford protein assay (Bradford, 1976).

2.5. Antioxidant activity

Cell oxidative stress was induced by H₂O₂ (100 μM). Three sets of cell treatments were performed: (a) cells exposed to H₂O₂ in PBS for 60 min; (b) cells pre-treated with HT or HVA (1–10 μM) 30 min before H₂O₂ treatment; (c) control cells. After H₂O₂ treatment, the cells were scraped on ice and centrifuged at 1200g at 4 °C for 5 min. After centrifugation pellets were separated from supernatants: the pellet was used for lipid analyses and for the evaluation of the protein concentration by the Bradford protein assay (Bradford, 1976), while MDA quantification was performed in the supernatant.

2.5.1. Determination of MDA

MDA concentration was determined as MDA-TBA adduct by HPLC (Templar et al., 1999). 0.25 mL of the medium were added with 1 mL of TCA (5%). After 10 min samples were centrifuged at 5000g for 15 min at 4 °C and 0.7 mL of the supernatant were added with 0.5 mL of TBA (0.6%) and incubated at 90 °C for 45 min. After centrifugation aliquots of the supernatant were injected into the HPLC-DAD system. Separation of MDA-TBA adduct was carried out using a Spherisorb column, Inertsil 5 ODS-2, 250 × 4.6 mm, with a mobile phase of 450 mM KH₂PO₄/Methanol (65/35, v/v) at a flow rate of 1 mL/min. Detection of the adduct peak was carried out at 532 nm. For each experiment a calibration curve was performed using standards (0–10 μM) of TEP.

2.5.2. Lipid extraction and determination of fatty acids, cholesterol and α-tocopherol

Total lipids were extracted from the cell pellet dissolved in 12 mL of CHCl₃/MeOH (2/1, v/v) solution as indicated by the Folch et al. procedure (Folch et al., 1957). Separation of α-tocopherol, cholesterol and free fatty acids was obtained by mild saponification (Bani et al., 1994) as follows: 6 mL of the CHCl₃ fraction, containing the lipids, from each sample was dried down and dissolved in 5 mL of ethanol; 100 μL of Desferal solution (25 mg/mL of H₂O₂), 1 mL of a water solution of ascorbic acid 25% (w/v), and 0.5 mL of 10 N KOH were added. The mixtures were left in the dark at room temperature for 14 h. After addition of 10 mL of n-hexane and 7 mL of H₂O₂ samples were centrifuged for 1 h at 900g. The hexane phase with

![Fig. 1. Structure of hydroxytyrosol and homovanillic alcohol.](image-url)
\(\alpha\)-tocopherol and cholesterol was collected, the dried residue was dissolved in 0.25 mL of MeOH and aliquots of the samples were injected into the HPLC system. After addition of further 10 mL of \(n\)-hexane to the mixtures, samples were acidified with 37% HCl to pH 3–4 and then centrifuged for 1 h at 900 g. The hexane phase with free fatty acids was collected, a part was evaporated and the residue was dissolved in 0.25 mL of CH\(_3\)CN with 0.14% (v/v) CH\(_3\)COOH. Aliquots of the samples were injected into the HPLC system. The recovery of fatty acids, cholesterol and \(\alpha\)-tocopherol was calculated by using an external standard mixture. All solvents evaporation was performed under vacuum. Separation of UFA and cholesterol was carried out with a HPLC-DAD system. Cholesterol, detected at 203 nm, and MeOH as mobile phase, at a flow rate of 1.5 mL/min. Electrochemical detector was set at an oxidizing potential of 0.6 V. Data were collected and analyzed using the Agilent Chemstation A.10.02. software, as detailed in a previous paper (Deiana et al., 1996). \(\alpha\)-Tocopherol amount was measured by electrochemical detection, using a Thermo Separation Products (Milan, Italy) P1000 pump equipped with an electrochemical detector INTRO (Antec Leyden, Leiden, The Netherlands). An automatic injector, Triathlon (Spark Holland BV, AJ Emmen, The Netherlands) was used. A C-18 Hewlett Packard ODS Hypersil column, 5 \(\mu\)m particle size, 100 \(\times\) 2.1 mm, was used with a mobile phase of MeOH/CH\(_3\)COONa 0.05 M pH 5.5 (95/5, v/v) at a flow rate of 0.3 mL/min. Electrochemical detector was set at an oxidizing potential of 0.6 V. Data were collected and analyzed using the Agilent Chemstation A.10.02. software.

2.6. Statistical analysis

INSTAT software (GraphPad software, San Diego, CA) was used to calculate the means and standard deviations of three independent experiments (\(n = 9\) for each sample/condition). One-way ANOVA was used to test whether the group means differed significantly.

3. Results

To determine HT metabolisation to HVA in LLC-PK1, analyses of the medium and the lysed cells were performed for different lengths of time 5 min-4 h with HT 250 \(\mu\)M, the highest non toxic concentration (data not shown). The quantity of HT added was mainly found in the medium, from 5 min of incubation, and the amount measured in the lysed cells was about 6.5%. After 4 h of incubation a peak, identified as HVA by comparison with the standard compound, was detected in the medium (Fig. 2).

In order to induce oxidative stress, LLC-PK1 cells were treated with H\(_2\)O\(_2\) (100 \(\mu\)M for 1 h); a concentration of 100 \(\mu\)M was chosen as the highest concentration able to induce a detectable lipid peroxidation process but not cell death (data not shown). H\(_2\)O\(_2\) exposure for 1 h resulted in MDA production: as shown in Fig. 3, a significant increase of MDA level in the culture medium of H\(_2\)O\(_2\) treated cells was observed compared with controls. Pretreatment with both phenolic compounds (30 min prior H\(_2\)O\(_2\) treatment) significantly inhibited MDA production, from 1 \(\mu\)M for HT and at 10 \(\mu\)M for HVA. To investigate membrane oxidative injury, the lipid fraction of the LLC-PK1 cells was extracted and the modification of the more oxidizable membrane lipids, UFA and cholesterol and the membrane antioxidant \(\alpha\)-tocopherol assessed. A preliminary set of experiments was performed to assess the effect of a 90 min treatment with HT or HVA (10 \(\mu\)M) on LLC-PK1 lipid profile and \(\alpha\)-tocopherol concentration. The two phenols did not affect the levels of fatty acids, cholesterol, and their oxidative products (HP and 7-keto), and \(\alpha\)-tocopherol in comparison with control cells. The major UFA present in the membrane were docosahexaenoic acid (22:6 \(n=3\)), eicosapentaenoic acid (20:5 \(n=3\)), arachidonic acid (20:4), eicosatrienoic acid (20:3 \(n=9\)), linoleic acid (18:2), oleic acid (18:1 \(n=9\)) and cis-vaccenic acid (18:1 \(n=7\)). H\(_2\)O\(_2\) treatment resulted in a significant depletion of all the measured UFA, around 20–30% of the initial level, as shown in Fig. 4. Pretreatment with the phenolic compounds preserved the concentration of all the measured UFA. From 5 \(\mu\)M of HT the amount of most of the measured UFA was significantly higher with respect to the non pretreated samples; HVA was less effective than HT, exerting a significant protection only at the highest concentration (10 \(\mu\)M). The depletion of the UFA concentration, expressed as total UFA in

![Fig. 2. Chromatograms showing hydroxytyrosol (HT) and homovanillic alcohol (HVA) standards (A) and the peaks detected in the medium after 4 h of incubation (B), registered at 280 nm, and HVA absorbance spectrum.](image_url)
Fig. 5, was inversely correlated with the increase of their major oxidation products, the fatty acids hydroperoxides (HP), indicating an ongoing oxidative process (Fig. 5). In the H$_2$O$_2$ treated cells the amount of HP was twice the controls, while in the samples pretreated with HT, at all the tested concentrations, HP value remained at the control level. The protective effect of HVA was also significant at 10 µM.

Fig. 6 shows the concentration of cholesterol and its major oxidation product 7-keto, measured in the HT or HVA pretreated and H$_2$O$_2$ treated cells with respect to the controls. Cholesterol level was significantly decreased after H$_2$O$_2$ treatment, with a reduction of 15%, and a related increase of 7-keto, three times the initial value, was also observed. Pretreatment with the phenolic compounds, significantly protected cholesterol from oxidation: there was no loss of cholesterol in the sample treated with 10 µM of both HT and HVA, and the concentration of 7-keto was significantly lower with respect to the oxidised samples from 1 µM and 5 µM respectively.

The concentration of the membrane antioxidant α-tocopherol was also measured and data are reported in Fig. 7: in the H$_2$O$_2$ treated cells, α-tocopherol was 70% of the control level. In the presence of both HT (from 5 µM) and HVA (at 10 µM) the loss of α-tocopherol was significantly reduced.

4. Discussion

During the last decade, a large body of evidence from both epidemiological and biochemical studies demonstrated that dietary intake of antioxidants play a protective role in human health, decreasing the incidence of several diseases. More recently a number of studies focused attention on the role of dietary antioxidant in kidney disease prevention (Singh et al., 2006). Oxidative stress constitutes the mechanism of production and progression of numerous renal diseases (Rodrigo and Rivera, 2002 and references therein). Injury may be partially caused by damage to DNA, cytoplasmic macromolecules, and to membrane lipids. The abundance of polyunsaturated fatty acids makes the kidney an organ particularly vulnerable to the lipid peroxidation process (Kubo et al., 1997), regarded as a key step common to the mechanism of a wide
range of renal diseases, as tubulointerstitial alterations (Martín-
Mateo et al., 1999). The kidney tubular epithelium is a major site
for the concentration and processing of a wide spectrum of injuri-
ous chemical species, such as LDL-ox, transition metals, hemoglo-
bin and myoglobin or potentially nephrotoxic drugs, and it is
thus exposed to a prooxidant environment (Rodrigo and Rivera,
2002 and references therein). Proximal tubular epithelial cells also
posses the ability to produce chemotactic cytochines, chemochines
and other inflammatory mediators (Ou and Natori, 1999). To study
the possibility that effects of antioxidants ameliorate tubulointer-
stitial damage, it is essential to be able to measure oxidative dam-
age accurately. Studies on oxidative damage in cell cultures have
become widely used to support antioxidant research prior to ani-
mal studies and human clinical trials. LLC-PK1 cells, an epithelial
cell line derived from pig kidney, retain characteristics of the pro-
ximal tubular epithelium (Perantoni and Berman, 1979), and there-
fore may be used as model to study renal toxicity. The generation
of $H_2O_2$ has been implicated in the pathogenesis of several forms of
acute tubular cell injury (Salahudeen, 1995).

Exposure of LLC-PK1 cells to $H_2O_2$ in vitro has been shown to re-
sult in ATP depletion, lipid peroxidation, DNA damage and cell
death (Meng and Reeves, 2000). Lipid peroxidation of cell
membranes results in the production of a number of secondary
products, including MDA, that are markers for the extent of cell
oxidation. In this study, together with the measurement of MDA le-
vel, we pointed out the effect of $H_2O_2$ on specific cell membrane li-
pid targets, the lipid components more susceptible to oxidation,
UFA and cholesterol and the membrane antioxidant $\alpha$-tocopherol,
measuring the concentration of HP and 7-keto, as biomarkers of
the oxidative damage. $H_2O_2$ treatment induced a significant in-
crease of the level of MDA, that matched the pattern of formation
of the more specific markers of lipid peroxidation: HP and 7-keto.
$H_2O_2$ exposure resulted in a disruption of the membrane structure,
that accounts for the loss of UFA, cholesterol and $\alpha$-tocopherol;

Fig. 5. Values of fatty acids hydroperoxides (HP) together with the total UFA measured in LLC-PK1 cells after 1 h incubation with $H_2O_2$ and treated with HT or HVA (1–10 $\mu$M), expressed as% of the control values (HP 0.35 ± 0.06 nmol/ng protein). *** = p < 0.001 versus controls, ** = p < 0.001; * = p < 0.01 versus $H_2O_2$ treated.

Fig. 6. Values of cholesterol and 7-ketocholesterol (7-keto) measured in LLC-PK1 cells after 1 h incubation with $H_2O_2$ and treated with HT or HVA (1–10 $\mu$M), expressed as% of the control values (70.88 ± 9.21 $\mu$g and 0.11 ± 0.02 $\mu$g/mg protein respectively). *** = p < 0.001 versus controls, ** = p < 0.001; * = p < 0.01 versus $H_2O_2$ treated.
membrane damage is a consequence of a lipid peroxidation process, as demonstrated by the significant production of oxidation products, as HP and 7-keto. Pretreatment with HT protected renal cells from oxidative damage: the level of membrane lipids was preserved and there was no significant detection of oxidation products. Our data show that HT is able to preserve the integrity of the biological membranes from detrimental oxidative process caused by free radicals in vitro. HVA, detected as metabolite of HT also in LLC-PK1 cells, exerts a protective action, significantly limiting the oxidative damage, although this effect is achieved at a higher concentration with respect to HT. Oxidative attack from the aqueous phase seems to be an important reaction for initiating membrane lipid peroxidation; extra virgin olive oil phenolic compounds can act as scavengers of aqueous radicals near the membrane and may also regenerate α-tocopherol (Paiva-Martins et al., 2003). It has been shown that HT can react with H2O2 (Maria De Lucia, 2006; O’Dowd et al., 2004), thus its protective effect against H2O2 induced damage in renal cells may be mainly due to a direct H2O2 scavenging property or to the scavenging of reactive species that are formed out of H2O2 (Rietjens et al., 2007), as observed in Caco-2 cultures (Manna et al., 1997) and human erythrocytes (Manna et al., 1999). HT is in fact mainly found outside the cells, where it may exert its protective action. However it permeates LLC-PK1 membranes, thus a protective role inside the cell can not be excluded. HVA could share the same mechanism of action, differing however for a methyl group, that affects its solubility, rendering HVA a more lipophilic compound. In this experimental system HT has superior antioxidant properties than HVA, lacking the catechol group, which high antioxidant activity can be explained by the high electron donating effect of the second hydroxyl group. However, in other experimental conditions, HVA was the most effective, compared to other simple phenols, in protecting LDL against copper-mediated oxidation and free radical scavenging ability as measured in the DPPH, FRAP and TEAC tests (Turner et al., 2005). HVA was also able to down-regulate the expression of adhesion molecules involved in early atherogenesis, through a mechanism not directly related to a scavenging effect (Dell’Agli et al., 2006). It is most likely that HT and HVA prevents H2O2 induced damage both by a direct radical scavenging action and via a more complex mechanism, involving signalling processes, that may be activated by H2O2 action. This is the first report on HVA protective effect against oxidative damage in a cell culture system and a comparison of HT and HVA antioxidant activity.

HT is present in a high concentration in extra virgin olive oil either in a free or esterified form, where it accounts for 70 to 80% of total phenolic fraction (Manna et al., 1999). It is interesting to note that many of the complex polyphenols present in olive oil (as secoiridoids derivatives) are not directly absorbed, but may be degraded in the gastrointestinal tract to yield increased amounts of free HT and tyrosol, which may be absorbed and exert biological effects (Corona et al., 2006). Human and animal study demonstrate that HT is rapidly absorbed and partly metabolized to HVA (de la Torre-Carbot et al., 2007), that is also present in extravirgin olive oil (Tuck et al., 2002). Our data demonstrated that both HT and HVA exert a protective action against oxidative damage in renal cells. We are following up these results with an animal model, in order to assess if the antioxidant action of HT is maintained also after in vivo metabolization to HVA.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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