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H_2O_2 oxidative activity: modulatory effect of hydroxytyrosol

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Azione ossidante del H_2O_2: effetto modulatorio dell'idrossitirosolo

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Hydroxytyrosol, lipid peroxidation, MAPK, Akt/PKB, LLC-PK1

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Summary
Hydroxytyrosol (HT) is the major o-diphenol present in extra-virgin olive oil, either in free or esterified form, once absorbed, is present in high amount in the kidney, where it may exert a proctetive action. In this study we monitored the ability of HT to protect renal cells (LLC-PK1) against oxidative damage induced by H_2O_2. The peroxidation of lipid represents a primary consequences of cellular oxidative stress, leading to biophysical changes that disrupt membrane and organelle function; moreover oxidative stress is a process that may stimulate cellular signalling pathways, usually associated with the promotion of cellular death. HT exerted a significant antioxidant action, inhibiting the production of fatty acids hydroperoxides and 7-ketocholesterol induced by H_2O_2 treatment and thus preserving the membrane lipids. It has been shown that oxidative stress in LLC-PK1 cells is related also to the changes in the phosphorylation state of pro-death signalling pathways ERK1/2 and the pro-survival signalling Akt/PKB. Pretreatment with HT is able to modulate H_2O_2-induced changes in the phosphorylation state of ERK1/2 and Akt/PKB. We suggest that one potential protective mechanism of olive oil polyphenols in kidney cells may be attributed to interactions with intracellular signalling pathways activated in response to oxidative stress.

Riassunto
L'idrossitirosolo (HT) è uno dei maggiori fenoli semplici presenti nell'olio extravergine d'oliva, presente in forma libera o coniugata; assunto con la dieta l'HT viene assorbito concentrandosi preferenzialmente a livello renale dove potrebbe esercitare un'azione protettiva. Lo scopo di questo lavoro è stato quello di valutare l'attività protettiva dell'HT contro il danno ossidativo indotto dal H_2O_2 in colture di cellule renali (LLC-PK1). La perossidazione lipidica rappresenta la conseguenza primaria dello stress ossidativo, ma lo stress ossidativo può agire anche indirettamente su vari pathways intracellulari e andare quindi ad interferire con i meccanismi di sopravvivenza o apoptosi. Il pretrattamento delle cellule LLC-PK1 con HT ha mostrato la capacità di prevenire la perossidazione lipidica indotta dal H_2O_2, inibendo in maniera significativa la forma-

zione di prodotti di ossidazione. È stato dimostrato che lo stress ossidativo è correlato con l'attivazione di ERK e della proteina chinasi B/Akt (Akt/PKB), la cui modulazione regola la sopravvivenza o l'apoptosi della cellula. L'HT sembra inibire il cambiamento di fosforilazione indotto dal H_2O_2, delle proteine considerate. I dati ottenuti confermano che l'HT nelle cellule renali, è in grado di esercitare un'azione protettiva, agendo sia da scavenger ma anche attraverso un meccanismo più complesso, che comporta l'interazione con i pathways intracellulari modulati dallo stress ossidativo.

Introduction

The role of ROS in the pathogenesis of different degenerative diseases was originally attributed to a "nonspecific" oxidative damage of biological targets leading to tissue degeneration and loss of function. But in the last years the discovery of specific genes and pathways affected by oxidants led to the hypothesis that ROS serve as intracellular messengers in gene regulatory and signal transduction pathways. In the other hand typical antioxidant molecules of nutritional interest have been considered able to modulate cellular responses to various stimuli interacting with ROS-mediated intracellular signaling (1).

In general, the chemical structure of phenols is compatible with a one-electron donor activity. They have been demonstrated to function as antioxidants in vitro in both cell cultures and cell-free systems by scavenging superoxide anion, singlet oxygen, and lipid peroxy-radicals and/or by stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing specie, but in some cases, the antioxidants have a role in interacting selectively within signalling cascades, such as tyrosine kinase, PI 3-kinase/Akt, PKC and MAP kinase pathways, that regulate cell survival following exposure to oxidative stress (1, 2).

H_2O_2 is one of the most important ROS in cellular systems,; It has been shown that the H_2O_2 induced oxidative cell damage is directly related to the lipid peroxidation process damage (3) but is also correlated with an indirect H_2O_2 action as H_2O_2 may induce an activation of MAPK and related gene expression (4).

Hydroxytyrosol (HT) is the major o-diphenol present in extra-virgin olive oil, either in free or esterified form, and once absorbed, is present in high amount in the kidney, where it may exert a proctetive action.

HT has been shown to protect cultured cells against H_2O_2 mediated damage: preincubation of intestinal Caco-2 cells with HT prevents H_2O_2-mediate oxidative damage (5); HT exerts a protective effect against the H_2O_2-induced oxidative hemolysis and MDA formation in red blood cells (6).

The aim of this study was to compare the classic antioxidant capacity of HT with its ability to modulate cellular signaling pathways, investigating its capacity to inhibit H_2O_2 induced oxidative damage in LLC-PK1 cells, a porcine kidney epithelial cell line that retains characteristics of the proximal tubular epithelium (7), where H_2O_2 treatment has been shown to initiate a lipid peroxidation process (8) and to induced changes in the phosphorylation state of pro-death signalling pathways ERK1/2 and the pro-survival signalling Akt/PKB (4).

Methods

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% CO_2. For experimental studies cells were plated at a density of about 5x104/mL and grown until reaching sub-confluence in Petri dishes o in the 6-well palte.

Antioxidant activity-protection of membrane lipids

Cell oxidative stress will be induced by H_2O_2; cells will be pretreated with different concentrations of HT 15 min befor H_2O_2 tretment (100μM; 1h), following treatment, cell pellets will be separated from supernatants: the supemataants will be used for MDA test and the pellet will be used for membrane lipid analyses.
MDA quantification - MDA levels in supernatants from treated cells will be measured with the TBARS test with HPLC quantification (9).
Lipid analyses - Total lipids will be extracted from the cell pellet by the Folch et al. procedure (10) and quantified as indicated by Chiang et al. (11). Separation of cholesterol and free fatty acids will be obtained by mild saponification (12). Analyses of unsaturated fatty acids, cholesterol, and their oxidative products, and vitamin A will be carried out with a HPLC equipped with a diode array detector (12).

Antioxidant activity-modulation of MAPK and Akt/PKB phosphorylation

Cell oxidative stress will be induced by treatment with H_2O_2; cells will be pretreated with different concentrations of HT 24h befor H_2O_2 tretment (25 uM;1h); following treatment, cell proteins will be extracted for Bradford quantification assay and Western immunoblotting analysis. Samples will be run on SDS-polyacrylamide gels and the proteins will be transferred to nitrocellulose membranes; the blots will be incubated with either anti-Akt, anti-phospho-Akt (Ser473), anti-p42/44 MAPK, anti-phospho-p42/44 MAPK (Thr202/Tyr204), anti-JNK, anti-phospho-JNK; resulting blots will be exposed to ECL® reagent and Hyperfilm-ECL for protein bands quantification (Quantity One software) (13).

Statistical analysis

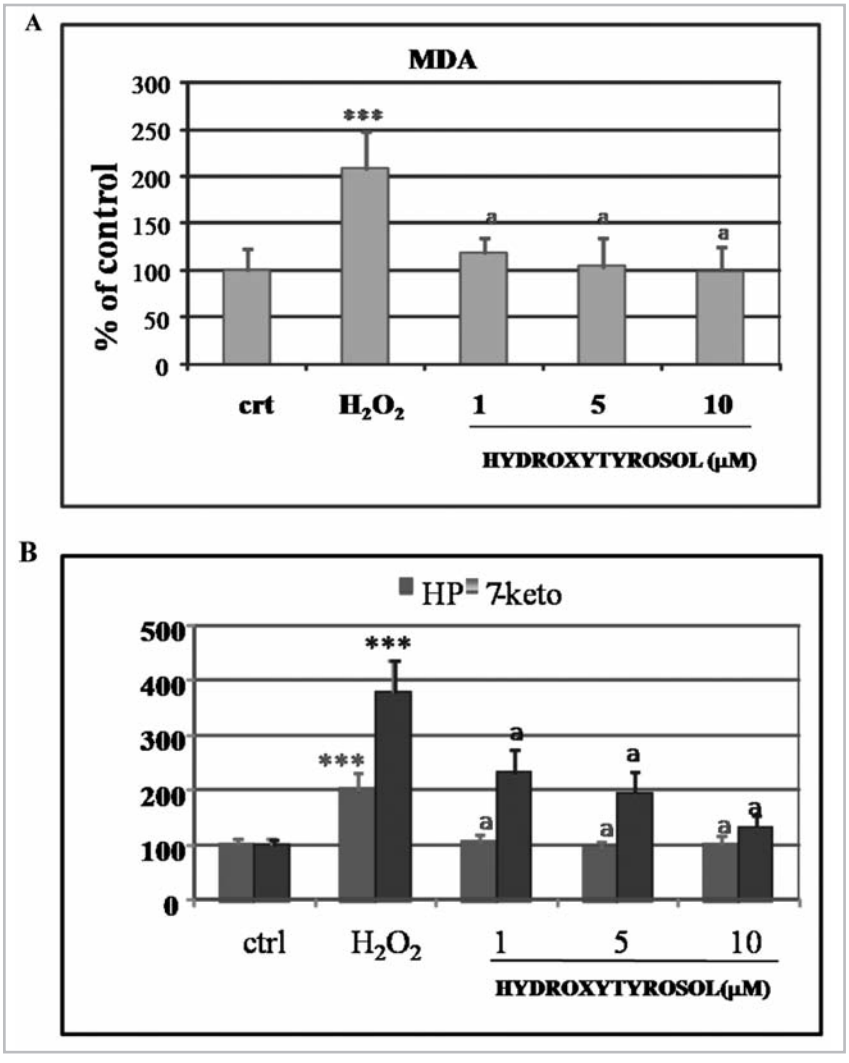
INSTAT software (GraphPad software, San Diego, CA) was used to calculate the means and standard deviations of three independent experiments (n=9 for each sample/condition). One-way ANOVA was used to test whether the group means differed significantly.

Results

Antioxidant activity-protection of membrane lipids.

In order to induce oxidative stress, LLC-PK1 cells were treated for 1 h with H_2O_2 100 μM, the highest concentration able to induce a detectable lipid peroxidation process but not cell death. H_2O_2 exposure resulted in MDA production: as shown in Fig. 1A, a significant increase of MDA level in the culture medium of H_2O_2 treated cells was observed compared with controls. Pretreatment with the phenolic compound (30 min prior H_2O_2 treatment) significantly inhibited MDA production, from 1 μM. To investigate membrane oxidative injury, the lipid fraction was extracted and the increasing of fatty acids hydroperoxides (HP) and 7-ketocholesterol (7-keto) measured. Figure 1B shows the concentration of 7-keto and HP, measured

Figura 1 - (A)Values of MDA measured in LLC-PK1 cells after 1 h incubation with H_2O_2 and treated with HT (1-10 μM), expressed as % of the control value (1.18±0.27 μmol/mg protein). (B). Values of unsaturated fatty acids hydroperoxide (HP) together with 7-ketocolesterol (7-keto) measured in LLC-PK1 cells after 1 h incubation with H_2O_2 and treated with HT (1-10 μM), expressed as % of the control values. *** = p<0.001 versus controls, a = p<0.001; b = p<0.01; c = p<0.05 versus H2O2 treated

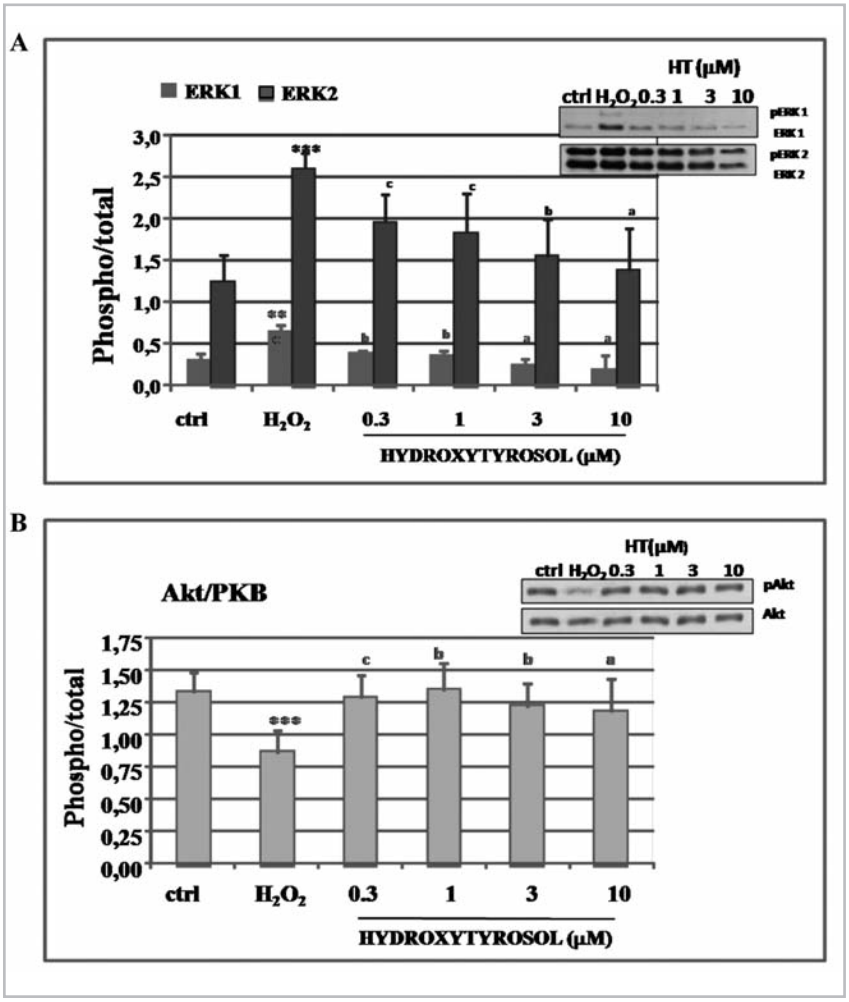


in the control and treated cells. The treatment with H_2O_2 induced an increase of HP. In the H_2O_2 treated cells the amount of HP was doubled with respect to the controls, while in the samples pre-treated with HT, at all the tested concentrations, HP value remained at the control level. 7-keto level was significantly increased after H_2O_2 treatment; pretreatment with HT significantly protected cholesterol from oxidation and the concentration of 7-keto was significantly lower from 1 μM.

Antioxidant activity-modulation of MAPK and Akt/PKB phosphorylation

In order to investigate whether HT could modulate H_2O_2-induced increases in ERK1/2 phosphorylation, cells were pre-treated with HT (0.3-10 μM, 24 h) prior to peroxide (25 μM; 1h) treatment (Fig. 2A). HT pretreatment prior to H_2O_2 exposure resulted in a significant inhibition in the peroxide-induced increases in ERK1/2 phosphorylation at all concentrations tested; H_2O_2 (25 μM; 1h) treatment resulted also in a marked decrease in Akt phosphorylation (Fig. 2B) with respect to the basal levels. Pre-treatment with HT (0.3-10 μM; 24h) prior to peroxide addition prevented this inhibition of Akt phosphorylation. Parallel immunoblots with a polyclonal antibody against total ERK1/2 and Akt protein levels were performed and indicated that there was no changes in total levels

Figura 2 - (A) . Modulation of H_2O_2-induced ERK phosphorylation by HT in kidney cells. Cells were treated with HT (0,3-10μM, 24 h) prior to addition of H_2O_2 (25μM, 1 h). (B). Modulation of H_2O_2-induced JNK phosphorylation by HT in kidney cells. Cells were treated with HT (0,3-10μM, 24 h) prior to addition of H_2O_2 (25μM, 1 h). *** = p<0.001 versus controls, a = p<0.001; b = p<0.01; c = p<0.05 versus H_2O_2 treated.



Conclusion

Recent literature suggests that molecules having a chemical structure compatible with a putative antioxidant capacity can actually "perform" activities and roles independent of such capacity, interacting with cellular functions at different levels (1).

Polyphenols have long been assumed to be 'antioxidants' that scavenge excessive, damaging, free radicals arising from normal metabolic processes. There is recent evidence that polyphenolics also have 'indirect' antioxidant effects through induction of endogenous protective enzymes (2).

For years, olive oil has been considered a product with many healthy properties; hydroxytyrosol (HT), the major o-diphenol present in extra-virgin olive oil, has been demonstrated in numerous studies to have different biological proprieties; in particular HT have been shown to possess a strong antioxidant activity in vitro and in vivo(14, 15).

This "antioxidant" activity has usually been considered beneficial for human health; in the present study, we investigated the protective effect of HT in kidney cells, as dual activity, the ability to preserve the membrane lipids against the lipid peroxidation induced by H_2O_2 and the ability to interact with the MAP kinase and PI3 kinase signalling pathways modulated by the H_2O_2.

In this study, we showed that the olive oil polyphenol, HT, evokes significant protection against H_2O_2 induced-cellular injury, in all the considerate parameters.

The peroxidation of lipids represents a primary consequences of cellular oxidative stress, leading to

biophysical changes that disrupt membrane and organelle function; moreover oxidative stress is a process that may stimulate cellular signalling pathways, generally associated with the promotion of cellular death.

H_2O_2 treatment induced a significant increase of the level of MDA, corresponding to the pattern of formation of the more specific markers of lipid peroxidation: HP and 7-keto. Pretreatment with HT protected renal cells from oxidative damage, as there was no significant detection of these oxidation products. H_2O_2 Oxidative damage in LLC-PK1 cells resulted also in the changes of the phosphorylation state of pro-death signalling pathways, ERK1/2 and the pro-survival signalling Akt/PKB; HT also is able to modulate H_2O_2-induced changes in the phosphorylation state of this signaling molecules

In summary, our results demonstrate that HT can exert a significant protection against H_2O_2 induced renal epithelial injury; these protective effects appear to be physiologically relevant, as HT has been found to be present in the circulation at low micromolar concentrations following oral intake.

These results suggest that HT, concentrating in the renal compartment, may exert a protective action against H_2O_2 mediated renal diseases, both through an interaction with intracellular signalling pathways and a direct protection against the lipid peroxidation process.

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