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Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells

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Hydroxytyrosol, tyrosol and caffeic acid effects on hydrogen peroxide-induced DNA damage, hydroperoxide generation and redox enzyme gene expression were studied in oxidative-stress-sensitive human prostate cells (PC3). Hydroxytyrosol led to lower levels of hydroperoxides, DNA damage, and mRNA levels of classic glutathione peroxidase (GPx) for all the studied concentrations. Only hydroxytyrosol was effective at low concentrations (10 μM). Tyrosol reduced DNA oxidation only at high (>50 μM) concentrations and increased hydroperoxides, GPx and phospholipid hydroperoxide GPx mRNA levels. Caffeic acid elicited effects between those of the other two phenolics. Results indicate that hydroxytyrosol is the only significant antioxidant phenolic in olive oil and may be the major component accounting for its beneficial properties. Tyrosol appeared to exhibit pro-oxidant effects (only at high concentrations) and caffeic acid was neutral. Both number and position of hydroxyl groups appear to play a role in the cellular effects of hydroxytyrosol.

Olive oil: Phenolic compounds: DNA damage: Gene expression

Increasing evidence suggests that the beneficial effects of olive oil intake on human health can be ascribed not only to elevated oleic acid content (Mataix *et al.* 1998) but also to antioxidant properties of its minor components, including phenolic compounds (Quiles *et al.* 1999*b*; Visioli *et al.* 2000*a*).

Phenolic compounds are found not only in olive oil but also in many plant species where they are present at high concentrations in many components of the 'Mediterranean diet', including fruit and vegetables (Ho *et al.* 1992; Manna *et al.* 1997). The average consumption of phenols probably exceeds 1 g/d, sufficient to have nutritional relevance if they are bioactive (Manna *et al.* 1997). Interest in olive-oil phenols is increasing as a consequence of their reported antioxidant properties (Visioli *et al.* 1998*a*) and the reported health benefits of olive oil in man. For example, protection against LDL oxidation and inhibition of platelet aggregation by hydroxytyrosol, one of the main olive-oil phenols, has been reported (Visioli *et al.* 1998*b*). Hydroxytyrosol also attenuates the cytokine-induced up regulation of vascular adhesion molecules in human endothelial cells in culture, which would reduce the likelihood of atherogenesis *in vivo* (Farquharson *et al.* 1999). Apart from their anti-atherogenic properties, these non-essential dietary components appear to elicit promising anti-carcinogenic effects (Della Regione *et al.* 2000; Owen *et al.* 2000) and prevent the release of arachidonic acid from membrane phospholipids through their inhibition of phospholipase A_2, thus reducing the production of chemotactic and inflammatory eicosanoids (Middleton & Kandaswami, 1992). Additionally, they are also inhibitors of lipoxygenase and cyclo-oxygenase activity *per se* (Laughton *et al.* 1991). Excessive eicosanoid production, especially prostaglandin E_2, is a major factor in angiogenesis and increased cancer progression; inhibition of prostaglandin E_2 production attenuates progression, particularly of colon cancers (Karmali, 1987). Finally, the anti-carcinogenic activity of phenols may be due not only to their antioxidant properties but also to their ability to reduce the bioavailability of food carcinogens and to inhibit their metabolic activation (Stavric, 1994). Other non-antioxidant effects of phenolics on gene regulation or enzyme activities cannot be precluded (Farquharson *et al.* 1999).

Despite the reported beneficial effects of phenolic compounds on health, there is still a lack of knowledge regarding the mechanism of action of these natural compounds.

Abbreviations: cGPx, classic glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase.

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For example, it is not clear what their bioavailability is in man or on what intracellular targets they exert their antioxidant and cell-regulatory effects. Furthermore, the relationship between their chemical structure, their antioxidant properties and their specific regulatory effects on the intrinsic antioxidant enzymes and various cellular control mechanisms are not clear at present.

The aim of the present study was to investigate: (i) the role of three different phenolic compounds, hydroxytyrosol, tyrosol and caffeic acid, on the oxidative damage of lipids and DNA in a cell-line derived from human prostate epithelium (PC3), this cell line having been chosen because we have shown it to be very sensitive to oxidative stress; (ii) the effect of these phenolics on gene regulation (mRNA expression) of intrinsic antioxidant enzymes (classic glutathione peroxidase (cGPx; *EC* 1.11.1.9) and phospholipid hydroperoxide glutathione peroxidase (PHGPx; *EC* 1.11.1.12)) in PC3 cells. The three phenolics were chosen (Fig. 1) because of their similarity in structure.

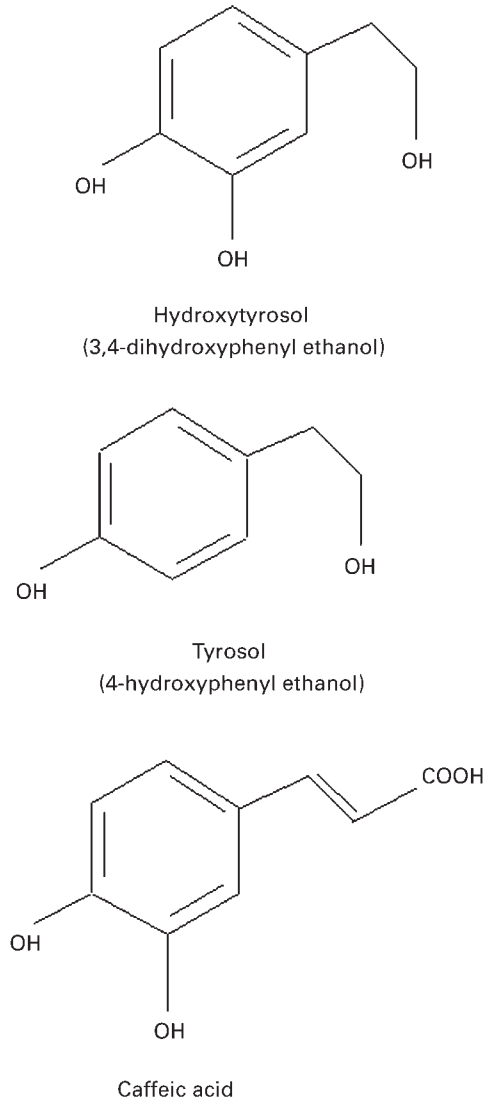

Fig. 1. Chemical structure of the three phenolic compounds used in the present study (hydroxytyrosol, tyrosol and caffeic acid).

Thus, hydroxytyrosol (3,4-dihydroxyphenyl ethanol), which is the main phenolic compound present in virgin olive oil (70–80 % of total phenolic fraction either in a free form or esterified as oleuropein; Baldioli *et al.* 1996), has an *o*-diphenolic structure and an ethylenic group. Tyrosol (4-hydroxyphenyl ethanol) has the same molecular structure as hydroxytyrosol but lacks the phenolic hydroxyl group. Caffeic acid has an *o*-diphenol structure but not an ethylenic group.

Understanding the underlying cell-regulatory mechanisms that are affected by these phenolic compounds may help to give a scientific basis to the reported health benefits of olive oil and the classical 'Mediterranean diet' as well as the structure–function relationship of phenolic compounds.

Materials and methods

Cell culture and treatment

PC3 human prostate cancer cells were grown in RPMI 1640 medium supplemented with 200μg streptomycin/ml, 200 IU penicillin/ml, 10 % (w/v) fetal calf serum and 100 μg filter-sterile sodium pyruvate/ml. Cells were maintained in an atmosphere of CO_2–air (5:95, v/v). Cells were passaged through subcultures by trypsinization at 0·05 % and seeded at the concentration of 4×10^4 cells/ml. Cells were treated for 24 h with 10, 50, 100 and 250 μM 3,4-dihydroxyphenylethanol (hydroxytyrosol), 4-hydroxyphenylethanol (tyrosol) or caffeic acid (see later for DNA oxidative-damage special conditions) by addition of the compounds to the medium in the form of 100-fold concentrated stock solutions of ethanol. All the cells were exposed to the same concentration of ethanol and these concentrations did not affect the mechanisms and end-points under investigation.

Cytotoxicity assay by flow cytometry

Propidium iodide was used in an exclusion experiment to assay the cytotoxicity of the various phenolics through the integrity of plasma membrane in the cells (Dengler *et al.* 1995). Propidium iodide fluoresces when it binds to DNA and is excluded from cells that have their plasma membrane integrity preserved. Consequently, the degree of fluorescence intensity correlates with the cytotoxic index of the compound being studied. After 24 h treatment, isolated cells were placed (10^6) in a plastic tube and suspended in 1 ml PBS. Propidium iodide (20 ml) was added (final concentration, 2 μg/ml) and cells were incubated for 5 min at room temperature in the dark before analysis. Flow cytometry was performed using a FACStar (Becton Dickinson, Mountain View, CA). Ar laser excitation was 50 mW at the 488 nm line. Red propidium-iodide fluorescence was detected above 620 nm. At least 15 000 cells (events) were analysed for each sample. Frequency distributions of propidium iodide concentration in the cells were obtained on a 4 decades fluorescence intensity scale (1024 channels) and descriptive statistics were performed. The median of the frequency distribution was chosen as representative of the propidium iodide level of the sample.

Oxidative DNA damage (the comet assay)

The comet assay was used to measure DNA strand breaks in the cells (Collins *et al.* 1996). DNA damage was assessed in cells subjected to different phenolic supplementation treatments for 24 h. Cells were then washed with PBS and divided into two equal portions. Each portion was treated for 5 min with 60 μM-H_2O_2 in incomplete medium to study the effect of the added phenolics on cell DNA-damage under acute conditions. Cells were also incubated for 5 min using only incomplete medium and no H_2O_2 stimulation in order to study the effects of the phenolics under baseline conditions. After isolation, cells were suspended in 1 % (w/v) low melting point agarose in PBS, pH 7·4, and pipetted onto microscope slides precoated with a layer of 1 % (w/v) normal melting point agarose (warmed to 37°C before use). The low melting point agarose was allowed to set at 4°C for 5 min and then the slides were immersed in a lysis solution (2·5 M-NaCl, 100 mM-EDTA, 10 mM-Tris at pH 10, 1 % (v/v) Triton X-100; Sigma, St Louis, MO, USA) at 4°C for 1 h to remove cellular proteins. Slides were then placed in an electrophoresis tank containing 0·3 M-NaOH and 1 mM-EDTA, pH 10 at 4°C for 40 min to allow the separation of the two DNA strands (alkaline unwinding). Electrophoresis was performed at 25 V, 300 mA for 30 min. The slides were then washed three times for 5 min each with a neutralizing solution (0·4 M-Tris, pH 7·5) at 4°C before staining with 1 mg 4,6-diamidino-2-phenylindole/ml.

4,6-Diamidino-2-phenylindole-stained nucleoids in each gel were examined under a u.v. microscope with an excitation filter of 435 nm and a magnification of 400. Analysis (blind) was performed using a charged couple device camera and Komet 3.0 image analysis program (Kinetic Imaging Ltd, Liverpool, UK). The percentage of DNA in the tail (mean of 100 comets per gel) is taken as a measure of DNA break frequency. The comet-like DNA formations were placed into five arbitrary classes (0, 1, 2, 3 and 4) according to the extent of DNA damage represented by a comet-like 'tail'. Each comet was assigned a value according to its class and the overall score for 100 comets ranged from 0 (100 % of comets being class 0) up to 400 (100 % of comets in class 4).

Determination of lipid peroxidation (hydroperoxides)

The ferrous oxide–xylenol orange method was used for determining hydroperoxides in the cells according to the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions (Jiang *et al.* 1991). After treatment with the different phenolic compounds, rubber-policeman isolated cells were incubated at 37°C for 30 min with the reagent in a water shaking bath. After centrifugation (2000 ***g*** for 5 s) the supernatant fractions were monitored at 560 nm using cumene hydroperoxide as standard.

Classic glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase mRNA levels

Total RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform procedure of Chomczynski & Sacchi (1987) and assessed by the A_{260}:A_{280} absorbance ratio. RNA species were then separated by electrophoresis through a denaturing 2·2 M-formaldehyde, 1·2 % (w/v) agarose gel and transferred to a nylon membrane by capillary blotting. RNA was fixed to the membrane by exposure to u.v. light and the membranes were stored dry until required. Membranes were pre-hybridized for at least 6 h at 42°C with 0·1 mg denatured salmon sperm DNA/ml in 50 % (v/v) formaldehyde, 10 % (w/v) dextran sulfate, 0·2 % (w/v) bovine serum albumin, 0·2 % (w/v) polyvinylpyrrilidone, 0·2 % (w/v) Ficoll, 0·1 % (w/v) sodium pyrophosphate, 1 % (w/v) SDS and 50 mM-Tris-HCl, pH 7·5. The DNA probes (30 ng) were labelled with [^{32}P]dcytosine triphosphate by random priming and hybridization carried out at 42°C for 24 h as described previously (Bermano *et al.* 1996). Membranes were then washed to remove non-specifically-bound probe, had two washes in 2 × saline-sodium citrate (SSC) (1 × SSC = 0·15 mol/l NaCl–0·015 mol/l sodium citrate) at room temperature for 5 min, followed by two washes at 65°C for 1 h in either 1 × SSC, 1 % (w/v) SDS (cGPx and PHGPx) or 0·2 × SSC, 1 % (w/v) SDS (18S rRNA) and a final wash in 0·1 × SSC at room temperature. Specific hybridization was then detected by electronic autoradiography using a Camberra Packard Instantimager (Packard, Pangbourne Berks, UK). After analysis membranes were washed in 0·1 % (w/v) SDS for 5–10 min at 95°C before rehybridization to other probes.

Quantification of the bound probe was carried out using the Instantimager (Packard) and results for each probe expressed per unit of hybridization achieved with the 18S rRNA probe. This allowed correction for any variation between loading of RNA on the gel or transfer to the nylon membrane.

cDNA probes and chemicals

3,4-Dihydroxyphenyl ethanol (hydroxytyrosol) was purchased from Cayman Chemical Company, Ann Arbor, MI, USA; 4-hydroxyphenyl ethanol (tyrosol) was purchased from Avocado Research Chemicals, Heysham, Lanc., UK. Propidium iodide was obtained from Molecular Probes, Europe, Leiden, Netherlands; cDNA probes for cGPx, PHGPx and 18S rRNA were a gift from Professor John Hesketh, Newcastle University, UK. All other products and reagents were of the highest grade available from commercial sources.

Statistical analysis

Results are presented as mean values with their standard errors for four experiments. Comparison of mean values between groups was assessed by one-way ANOVA followed by a *post hoc* Duncan's test. Previously, all variables were tested for normal and homogeneous variance by Levene test. When a variable was found not normal, it was log-transformed and reanalysed. P values of less than 0·05 were considered significant. Data were analysed using SPSS statistical software package (SPSS for Windows, 9.0.1, 1999; SPSS Inc., Chicago, IL, USA).

Results

Cell viability and cytotoxicity

The flow cytometry study of propidium iodide incorporation and exclusion did not show any differences in cell viability among PC3 cells treated with hydroxytyrosol, caffeic acid or tyrosol at the different concentrations with regard to the control (data not shown). This shows that even at pharmacological concentrations these phenolics do not appear to be cytotoxic.

DNA oxidative damage (comet assay)

Control, non-stimulated cells showed the lowest values of DNA damage (analysed by the comet assay) and this was taken as the 100 % value (Fig. 2). No differences with regard to control were found for PC3 cells in the absence of H_2O_2 when treated with different concentrations of hydroxytyrosol, tyrosol and caffeic acid (values ranged from 88 (SEM 15) to 115 (SEM 22)) (data not shown). Cells without added phenolics, but treated with 60 μM-H_2O_2, had a 12-fold increase in DNA damage compared with control cells. Fig. 2 also shows the level of oxidative damage in the DNA of cells stimulated with 60 μM-H_2O_2 for 5 min analysed by the comet assay. DNA damage was reduced dramatically in cells with increasing concentrations of hydroxytyrosol and incubated with H_2O_2. Decreased damage was proportional to the concentration of the phenolic compound, with the lowest damage observed for 250 μM-hydroxytyrosol. For tyrosol, only 100 and 250 μM concentrations were able to decrease DNA damage of peroxide-treated cells but this decrease was less than that found with the same concentrations of hydroxytyrosol-treated cells. Cells treated with 50, 100 and 250 μM-caffeic acid also exhibited a dose-dependent decrease in DNA damage in PC3 cells treated with H_2O_2; the caffeic acid effectiveness was higher than tyrosol but lower than hydroxytyrosol.

Lipid peroxidation (hydroperoxides generation)

Fig. 3 depicts the hydroperoxide production in PC3 cells treated for 24 h with hydroxytyrosol, tyrosol or caffeic acid. Irrespective of the concentration used (10, 50, 100 or 250 μM) cells treated with hydroxytyrosol produced significantly less hydroperoxides than control cells (100 %) and those treated with tyrosol or caffeic acid; increasing concentrations of hydroxytyrosol above the 10 μM level did not result in further reductions in peroxides. Tyrosol-treated cells produced the highest proportion of hydroperoxides and levels were generally greater than in controls; again, values were independent of phenolic concentrations. Caffeic acid-treated cells produced a similar proportion of hydroperoxides as control cells; the proportion was greater than that produced by cells treated with hydroxytyrosol and lower than those treated with tyrosol and was independent of the phenolic concentrations.

Classic glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase mRNA levels

The stable transcription rate for cGPx mRNA is shown in Fig. 4. Hydroxytyrosol-treated cells exhibited significantly lower mRNA abundance than control cells (100 %) at all concentrations. The 50, 100 and 250 μM treatments elicited similar but lower effects than that of the 10 μM treatment. For tyrosol-treated cells, all concentrations except 10 μM elicited a higher mRNA abundance than control cells and

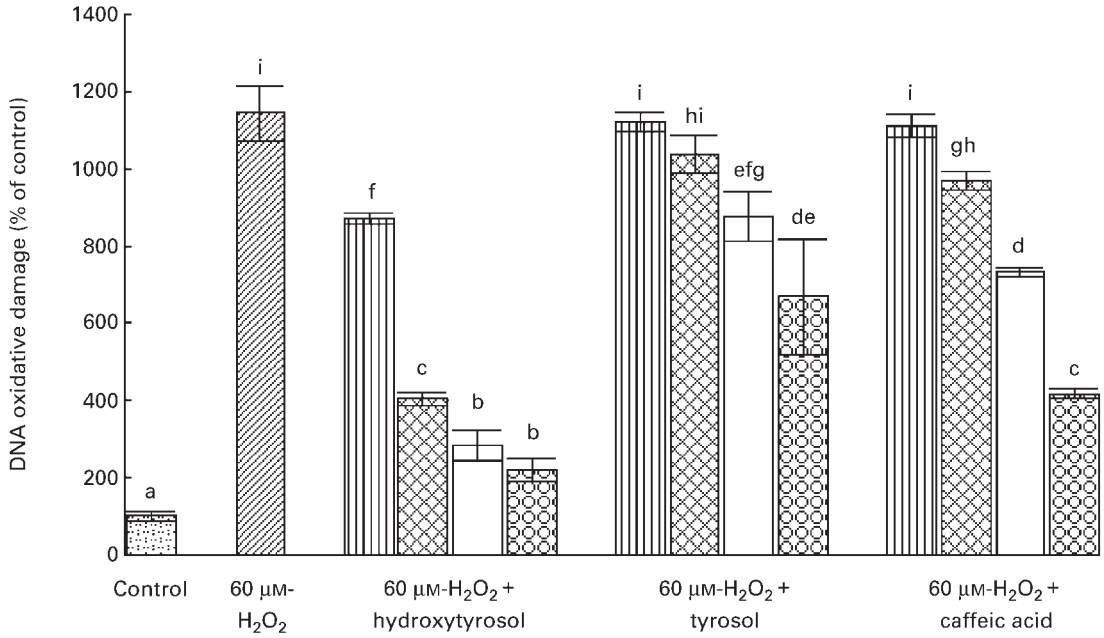

Fig. 2. Effect of 24 h incubation with different concentrations of hydroxytyrosol, tyrosol and caffeic acid on DNA oxidative damage in PC3 cells. (▥), 10 μM; (▩), 50 μM; (□), 100 μM; (▨), 250 μM. Before being harvested, cells were treated for 5 min with 60μM-H_2O_2. Results are mean values of four different experiments; standard errors of the mean are represented by vertical bars. Values with different superscripts are significantly different ($P<0{\cdot}05$) according to a one-way ANOVA followed by Duncan's test.

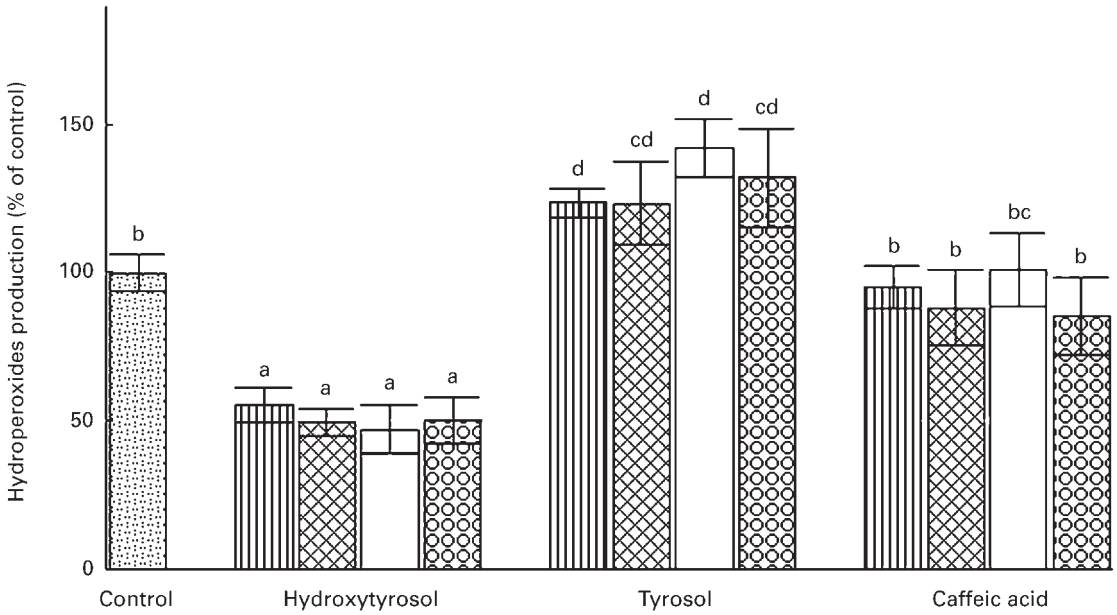

Fig. 3. Effect of 24 h incubation with different concentrations of hydroxytyrosol, tyrosol and caffeic acid on the production of lipid hydroperoxides in PC3 cells. (▥), 10 μM; (▩), 50 μM; (□), 100 μM; (▨), 250 μM. Results are mean values of four different experiments; standard errors of the mean are represented by vertical bars. Values with different superscripts are significantly different ($P<0·05$) according to a one-way ANOVA followed by Duncan's test.

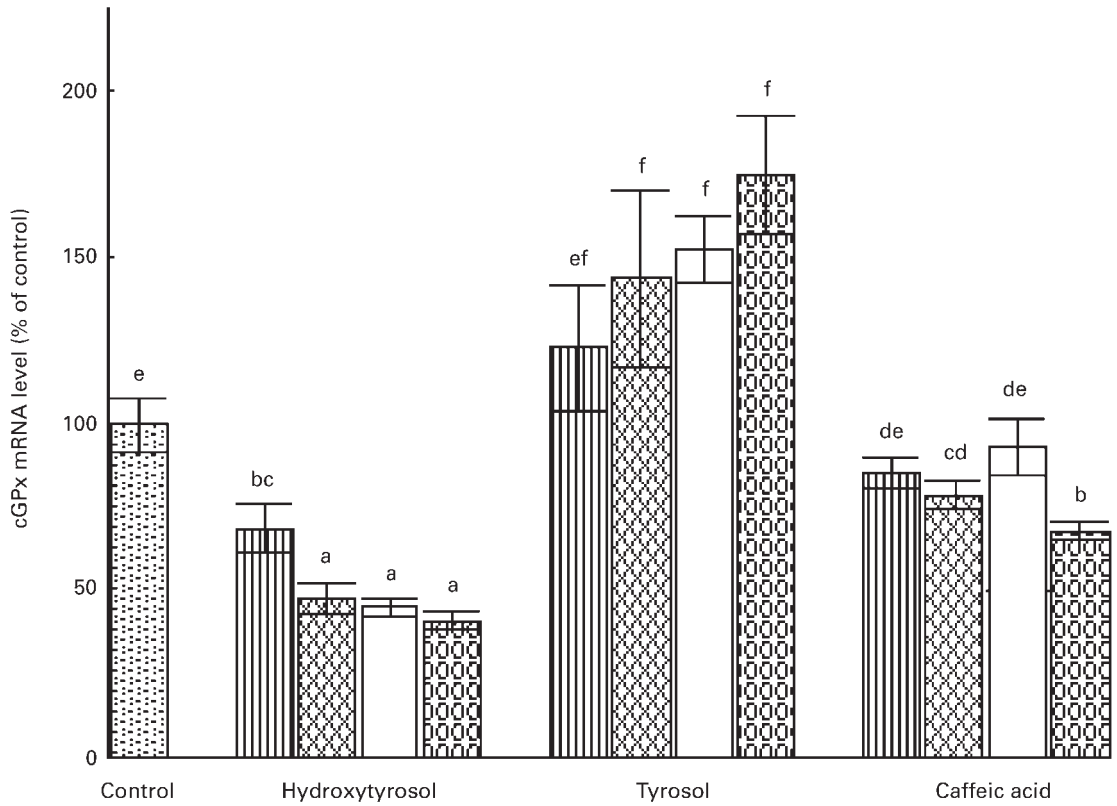

Fig. 4. Effect of 24 h incubation with different concentrations of hydroxytyrosol, tyrosol and caffeic acid on the relative mRNA levels of classic glutathione peroxidase (cGPx) in PC3 cells. (▥), 10 μM; (▩), 50 μM; (□), 100 μM; (▨), 250 μM. Results are mean values of four different experiments; standard errors of the mean are represented by vertical bars. Values with different superscripts are significantly different ($P<0·05$) according to a one-way ANOVA followed by Duncan's test.

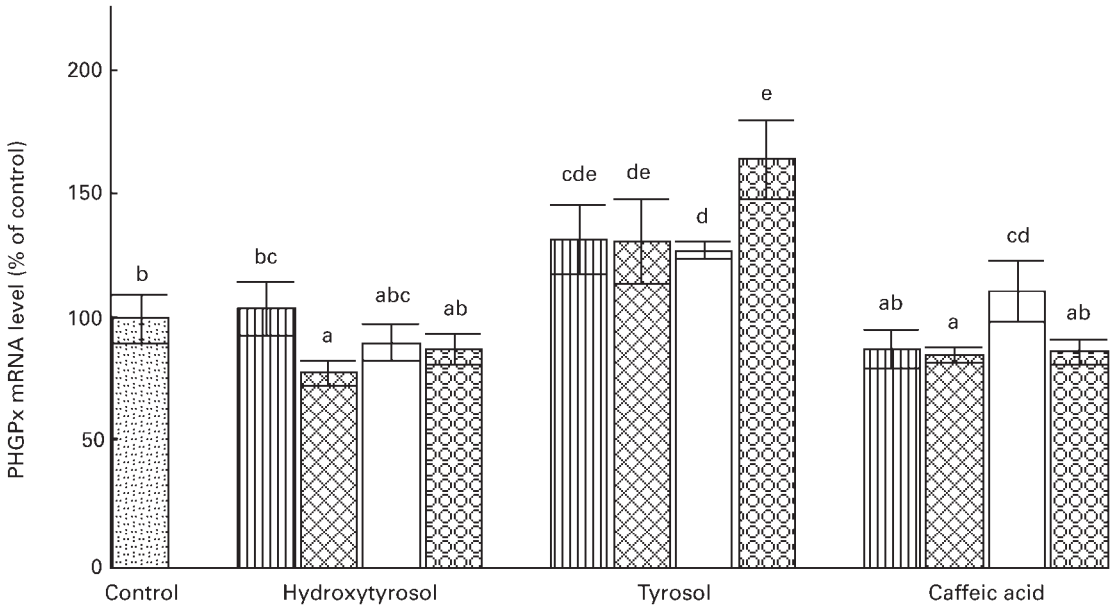

Fig. 5. Effect of 24 h incubation with different concentrations of hydroxytyrosol, tyrosol and caffeic acid on the relative mRNA levels of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in PC3 cells. (▥), 10 μM; (▨), 50 μM; (□), 100 μM; (▩), 250 μM. Results are mean values of four different experiments; standard errors of the mean are represented by vertical bars. Values with different superscripts are significantly different ($P<0·05$) according to a one-way ANOVA followed by Duncan's test.

the increase was similar for the different concentrations. In the caffeic acid-treated cells, 10 and 100 μM did not elicit changes in cGPx mRNA abundance compared with control cells, but 50 and 250 μM concentrations elicited an attenuation of abundance compared with control cells; values were not as low as those observed with hydroxytyrosol.

Fig. 5 shows results for gene expression of PHGPx (mRNA) in cells treated with different concentrations of phenolics. With hydroxytyrosol treatments, only the 50 μM concentration was able to elicit lower mRNA abundance than control cells (100 %). All other concentrations exhibited similar mRNA abundance to that in control cells. All tyrosol concentrations elicited a higher mRNA abundance than control cells; all concentrations resulted in a similar abundance except 250 μM, which gave the highest value. The results obtained after caffeic acid treatment of PC3 cells for 24 h were also not greatly different from control values. Concentrations of 10 and 250 μM did not elicit any changes in mRNA abundance compared with control cells whilst 50 μM elicited a slightly lower and 100 μM a somewhat higher abundance compared with controls.

Discussion

It is increasingly apparent that the reported health benefits of dietary olive oil in the classical 'Mediterranean diet' may not be entirely due to the lipid component of the oil and that minor components such as mono-phenolics may play an important role (Mattson & Grundy, 1985; Keys & Keys, 1995; Willett, 1997; Mataix *et al.* 1998; Quiles *et al.* 1999*a*; Ramírez-Tortosa *et al.* 1999). Several studies have been conducted to elucidate the contribution of the phenolic components in virgin olive oil to the positive health effects attributed to the oil *per se*. However, to the best of our knowledge, no studies have been conducted that assess the efficacy of olive oil phenolics in regulating the gene expression of intrinsic antioxidant enzymes such as mRNA abundance of cGPx and PHGPx. Similarly, many questions relating to the intracellular site of action of these phenolics and their effectiveness in protecting cells against oxidative damage to DNA remain to be answered.

The present work describes the effectiveness of three olive oil phenolic derivatives with a similar structure (hydroxytyrosol, tyrosol and caffeic acid) that are present in virgin olive oil. They occur in variable concentrations (depending on virgin olive oil variety, geographical origin, harvest time, storage conditions, etc.), ranging from 2·6–27 mg/kg for hydroxytyrosol, 2·98–61·5 mg/kg for tyrosol and 0·1–14·7 mg/kg for caffeic acid (Montedoro *et al.* 1992; Baldioli *et al.* 1996; Cinquanta *et al.* 1997; Owen *et al.* 2000). Although similar in structure, they differ in the number and position of their hydroxyl groups, which may be important in modulating the oxidative DNA damage and oxidant-induced gene expression of redox enzymes in human prostate cells elicited by H_2O_2. A recent study reported decreased tissue lipid peroxidation and increased tissue glutathione peroxidase activity in rabbits supplemented with olive oil in their diets, which suggested opposing anti- and pro-oxidant effects. These authors did not report changes in glutathione peroxidase gene expression (De la Cruz *et al.* 2000). These animal studies differ from the present cell studies in that the hydroxytyrosol in the current study elicited both decreased hydroperoxide formation and reduced glutathione peroxidase mRNA abundance, which appears more logical since cGPx is induced by oxidative stress. The increased

cGPx observed in the animal studies compared with the decrease found in cells could be due to time-lapse differences between mRNA increase and the expression and the availability and durability of the enzyme protein.

The negligible DNA damage and lack of effect of the phenolics in cells that were not stimulated with H_2O_2 is normal considering that cells usually contain a variety of different antioxidant systems to protect their genetic material from damage by reactive oxygen species under physiological conditions (Sies, 1993). However, after induction of damage with 60 μM-H_2O_2 for 5 min the phenolics were able to protect cells from oxidative DNA damage. It was also evident from the results that the degree of protection afforded by these phenolics depended on their structure and the concentration used. Previous studies have clearly demonstrated a lack of protection against oxidative stress by tyrosol when compared with hydroxytyrosol in a wide variety of systems and oxidative conditions (Visioli & Galli, 1998; Manna *et al.* 1999). However, there are no comparisons in the literature between hydroxytyrosol, tyrosol and other phenolics with regard to oxidative DNA damage and the regulation of redox enzymes at the level of gene transcription in human prostate cells. The present study showed that hydroxytyrosol effectively protected prostate cells against the DNA damaging effects of H_2O_2. Concentrations as low as 10 μM resulted in a 23 % reduction in damage whilst the highest concentration (250 μM) resulted in an 80 % reduction compared with H_2O_2-stimulated cells not treated with the phenolic.

To the best of our knowledge there are no definitive studies relating serum levels of olive oil phenolics to olive oil intake in human volunteers. This makes it difficult to estimate the likely physiological concentrations that might occur in plasma and tissues (and cells). However, Visioli *et al.* (2000*a*,*b*) reported that the absorption of hydroxytyrosol and tyrosol in human subjects (determined in 24 h urine samples) was 30–60 % and 20–22 % respectively of total intake. Owen *et al.* (2000) have estimated that virgin olive oil has 14·42 (SEM 3·01) mg hydroxytyrosol/kg and 27·45 (SEM 4·05) mg tyrosol/kg. These data must be carefully considered due to the high degree of variation among virgin olive oil samples (and keeping in mind that hydroxytyrosol is also present in the form of oleuropein), but it can be useful to calculate average concentrations in oils and thereby estimate average intakes in man. Thus, it is possible to assume concentrations of 81·7 μM hydroxytyrosol in virgin olive oil and 175·5 μM tyrosol. If the highest degree of absorption proposed by Visioli *et al.* (2000*a*,*b*) is considered, then up to 49 μM hydroxytyrosol and 38·6 μM tyrosol from virgin olive oil might be absorbed. The 10, 50 and even 100 μM concentrations of phenolics used in the present study are probably within the physiological range, but the 250 μM probably exceeds this range and could be regarded as being in the pharmacological range. This would also pertain to some of the published studies *in vitro* in which concentrations of 500 and 1000 μM phenolics are used (for example, Petroni *et al.* 1997; Deiana *et al.* 1999). It is not known to what extent the phenolics present in olive oil can accumulate in different tissues. This could result in localized variations in their concentration, which would not reflect their plasma concentrations.

In the case of tyrosol, only 100 μM with a 23 % of reduction of the damage and 250 μM with a 40 % of reduction was able to protect DNA from H_2O_2-damage. Caffeic acid reduction of DNA damage was intermediate between that elicited by hydroxytyrosol and by tyrosol (i.e. 15, 35 and 63 % of reduction for 50, 100 and 250 μM dosages respectively).

Functional differences between hydroxytyrosol and tyrosol have been attributed to the presence of only a single hydroxyl group in tyrosol compared with two of these groups present in hydroxytyrosol (Manna *et al.* 1999). Masella *et al.* (1999) suggests that the phenol chemical structure considerably influences the antioxidant activity as a consequence of both steric factors and those related to position and type of functional groups on the phenol ring. These authors stated that the antioxidant activity of biophenols depends mainly on the number of hydroxyl groups in the molecule. They also suggested that insertion of an ethylenic group between a phenyl ring and the carboxylate group ensured greater H-donating ability and subsequent radical stabilization. These considerations are in agreement with the present functional results in relation to the structures of hydroxytyrosol, tyrosol and caffeic acid. Hydroxytyrosol has two hydroxyl groups and an ethylenic group and exhibited the most significant antioxidant and gene regulatory effects. Tyrosol has the ethylenic group but only a single 4-hydroxyl group and caffeic acid has two hydroxyl groups (3,4 OH) but no ethylenic group. Clearly, all three hydroxyl groups together are important for activity with the 3-hydroxy playing a key role in antioxidant function.

The efficacy of some plant-derived phenolics as inhibitors of mutational and precarcinogenic events in biological systems has been reported (Newmark, 1987) and Deiana *et al.* (1999) observed that hydroxytyrosol protected neuronal hybridoma cells against the peroxynitrite-dependent nitration of tyrosine and DNA damage by peroxynitrite *in vitro*. These beneficial effects of hydroxytyrosol against oxidation of DNA and its general antioxidant properties are confirmed and extended in the present study and are further augmented by the inhibitory effects on redox enzyme gene expression. The lower effectiveness of caffeic acid and the promotion of oxidative stress by tyrosol probably relates to their specific structure (see earlier). However, since hydroxytyrosol is absorbed to a greater extent than other virgin olive oil phenolics (Visioli *et al.* 2000*b*) and has the highest antioxidant activity, it is likely that its antioxidant and redox inhibitory effects will predominate. It cannot, however, be precluded that the other phenolics contribute to the beneficial properties of this dietary fat through non-antioxidant mechanisms (Sies, 1993), especially after recent studies confirming their absorption and availability to tissues (Manna *et al.* 2000; Visioli *et al.* 2000*b*).

In order to test the antioxidant capacity of our phenolic molecules we also determined a marker of lipid peroxidation in the cells as the levels of lipid hydroperoxides. In contrast to what happened with DNA damage, baseline conditions were enough to promote differences in lipid

peroxidation. It has previously been demonstrated that under normal conditions, conventional cell culture media may promote oxidative stress because of their antioxidant deficiencies (Leist *et al.* 1996). Present results clearly show that hydroxytyrosol-treated cells produced 50 % less hydroperoxide than control cells, irrespective of hydroxytyrosol concentrations. This lack of effect of phenolic dose–response effect on hydroperoxide formation was also found for tyrosol and caffeic acid. The pro-oxidant and antioxidant effects of tyrosol and hydroxytyrosol respectively have been previously described using other models and other markers of lipid peroxidation (Manna *et al.* 1997). No data from experiments with caffeic acid have been reported to date and at the present moment it is not possible to explain the absence of a concentration effect of these phenolics on hydroperoxide formation in the PC3 cells.

As a part of the test to study the putative antioxidant capacity of the assayed phenolic compounds, we studied the relative mRNA abundance (gene expression) of cGPx and PHGPx in prostate cells treated with the earlier-mentioned molecules. The attention has been focused on these antioxidant enzymes because of their particular subcellular distribution. cGPx is present in cytosol and the mitochondrial matrix, i.e. hydrophilic environments (Halliwell & Gutteridge, 1999). PHGPx is a monomeric protein of relative molecular mass 19 000 present in cell membranes that can reduce esterified fatty acid and cholesterol hydroperoxides. It can act upon these oxidized fatty acids directly within membranes and lipoproteins (unlike cGPx), reducing them to alcohols (Arai *et al.* 1999). Because of their regulation by oxidative stress, these enzymes may help us to elucidate the antioxidant properties of the olive oil phenolics and, very importantly, to elucidate their site of action.

As it happened for the lipid peroxidation experiment, baseline conditions were enough to produce differences in terms of RNA transcription for the assayed enzymes. Fig. 4 shows hydroxytyrosol produced less expression of cGPx than caffeic acid and especially than tyrosol. As for hydroperoxides, all the dosages (except 10 μM for hydroxytyrosol and 250 μM for caffeic acid) produced similar levels. The present findings clearly show that cells treated with hydroxytyrosol were more protected against oxidative stress than control cells or those treated with either tyrosol or caffeic acid and the response was more dependent on the chemical structure than on the dosage. Hydroxytyrosol reduced cGPx mRNA abundance, which correlated with the lowest level of hydroperoxides indicating a high degree of protection of DNA from oxidative damage. Neither hydroxytyrosol nor caffeic acid affected PHGPx mRNA abundance but tyrosol produced a small increase (15 %) in expression compared with control cells. This indicates that the phenolics affected cGPx mRNA to a greater extent than PHGPx mRNA, which may reflect differential regulation of the specific genes by these phenolics. These findings relating to the differential regulation of cGPx and PHGPx transcription by olive oil phenolics are novel and are indicative of specific nutrient–gene interaction, which could explain, at least in part, the beneficial effects of dietary olive oil. Furthermore, these findings suggest that these phenolics may elicit differences in the availability (amount) of cytosolic and membrane-bound redox enzymes (Manna *et al.* 2000).

In summary, results from the present study suggest that hydroxytyrosol and caffeic acid, two phenolic compounds present in the non-glyceride fraction of virgin olive oil, may act as antioxidants protecting DNA and lipids against oxidative damage. Hydroxytyrosol is more efficient than caffeic acid. However, tyrosol appears to be a pro-oxidant, albeit at high concentrations. In terms of chemical structure, differences in efficacy may be due to the presence of two hydroxyl groups in an *o*-position together with an ethylenic group in hydroxytyrosol. Caffeic acid, which only has two hydroxyl groups, has a lower antioxidant capability and finally, tyrosol, which has a single hydroxyl group plus the ethylenic group, has no antioxidant capacity and may even act as a pro-oxidant. Since hydroxytyrosol is the most active phenolic compound in virgin olive oil and has a higher degree of absorption than molecules such as tyrosol (Visioli *et al.* 2000*b*), it is conceivable that its antioxidant effects predominate. This would explain the reported beneficial effects on health of virgin olive oil consumption in Mediterranean countries. These phenolics are also able to modify the gene expression of cGPx more than of PHGPx suggesting that they act better under hydrophilic atmospheres than in membrane structures, which may be an important clue concerning the subcellular distribution of virgin olive oil phenolics.

Finally, 100 and 250 μM hydroxytyrosol decreased cell proliferation with lower cell numbers observed (74·1 and 53·5 % relative to the control, respectively), whereas tyrosol increased cell proliferation at 10 and 50 μM tyrosol with higher cell number (148·6 and 171·5 % relative to the control, respectively) being observed. This finding is indicative of increased apoptosis for the higher concentrations of hydroxytyrosol. Similar effects on cell proliferation were found by Della Regione *et al.* (2000) studying human myeloid leukemia HL60 cells. Thus, it is possible to speculate that 100 and 250 μM hydroxytyrosol could be pro-apoptotic or at least anti-proliferative, which could affect tumour growth and development *in vivo*. By contrast, however, tyrosol could increase cell proliferation, which would be expected to enhance tumorigenesis. Interestingly (data not shown), the anti-proliferative effects elicited by hydroxytyrosol in oxidative-stress sensitive PC3 cells were not found in less sensitive non-cancer prostate cells (PNT2). The relevance of these findings to prostate cancer and its prevention or amelioration is not clear at present and requires further study with emphasis on the expression of apoptotic genes and cell death *per se*.

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