Nutrient-Gene Interactions

Extra Virgin Olive Oil Biophenols Inhibit Cell-Mediated Oxidation of LDL by Increasing the mRNA Transcription of Glutathione-Related Enzymes[1]

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ABSTRACT It has been reported that oxidized LDL (oxLDL) are involved in the pathogenesis of atherosclerosis, and that macrophages as well as other cells of the arterial wall can oxidize LDL in vitro, depending on the balance between intracellular prooxidant generation and antioxidant defense efficiency. Because of their possible beneficial role in the prevention of atherosclerosis and other oxidative stress–associated diseases, phenolic compounds naturally occurring in vegetables, fruits, and beverages are receiving increased attention. In the present work, we investigated the mechanisms underlying the protective effect exerted by extra virgin olive oil biophenols, namely, protocatechuic acid and oleuropein, on LDL oxidation mediated by murine J774 A.1 macrophage-like cells. The biophenols were added to the cells with LDL and left in the medium during the entire experimental period, or for a period of 2 h and then removed from the medium before the addition of LDL. The effect of biophenols alone was also tested. In both experimental procedures, these antioxidants had the following effects: *1*) completely prevented the J774 A.1-mediated oxidation of LDL; *2*) counteracted the time-dependent variations in intracellular redox balance, inhibiting the production of $O_2^{\cdot-}$ and H_2O_2 and the decrease in glutathione (GSH) content; *3*) restored glutathione reductase (GR) and peroxidase (GPx) activities; and *4*) restored the mRNA expression of γ-glutamylcisteine synthetase (γGCS), GR, and GPx to control values. More importantly, we observed significant overtranscription and increased activities of two antioxidative enzymes, GPx and GR, compared with controls when the biophenols were present in the medium for 2 h and then removed before LDL exposure, or when the cells were exposed to the antioxidants alone for up to 24 h. Our findings suggest that the activation of mRNA transcription of GSH-related enzymes represents an important mechanism in phenolic antioxidative action. J. Nutr. 134: 785–791, 2004.

KEY WORDS: • *oxidized LDL* • *biophenols* • *olive oil* • *glutathione* • *oxidative stress*

Oxidized LDL (oxLDL)[4] have been reported to play an important role in the pathogenesis of atherosclerosis (1). When subjected to oxidative stress, the cells of the arterial wall, i.e., endothelial cells, smooth muscle cells, and macrophages, can oxidize LDL in vitro (2,3). Macrophage-mediated oxidation of LDL depends on the intracellular balance between cellular oxygenase products, i.e., superoxide anions, and antioxidant content such as glutathione (GSH) and its related enzymes glutathione peroxidase (GPx), glutathione reductase (GR), and γ-glutamilcysteine synthetase (γGCS) (4–6).

Phenolic antioxidants, naturally occurring in vegetables, fruits, and beverages (7) such as tea and red wine, may play a key role in the prevention of cardiovascular diseases (8). Phenol intake has been reported to be inversely related to mortality from coronary heart disease and to the incidence of myocardial infarction (9–11). Experimental studies demonstrated that LDL oxidation is substantially reduced by consumption of several phenolic compounds (12,13). Moreover, some flavonoids can modulate the activity of enzymes involved in the cell-mediated oxidation of LDL and in the preservation of GSH (14–17).

Extra virgin olive oil, the typical added fat of the Mediterranean diet, has been associated with a reduced incidence of risk factors for coronary heart disease. This is partly due to the high amount of monounsaturated fatty acids (18), but it is likely to be also the result of the presence of phenolic compounds that have strong antioxidative power. Although several phenolic compounds, e.g., those in green tea and licorice root, were demonstrated to inhibit macrophage cell-mediated oxidation of LDL (19,20), to our knowledge, little information exists concerning similar activity or related mechanisms for the biophenols contained in extra virgin olive oil.

The aim of this study, therefore, was to analyze the effects of two phenolic compounds contained in extra virgin olive oil,

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[4] Abbreviations used: CM-H2-DCFDA, 5-chloromethyl-2′,7′-dichlorodihydrofluoresceindiacetate; DHE, dihydroetidium; DHR 123, dihydrorhodamine 123; FCS, fetal calf serum; γGCS, γ-glutamilcysteine synthetase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; oxLDL, oxidized LDL; PI, propidium iodide; REM, relative electrophoretic mobility; ROS, reactive oxygen species.

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i.e., protocatechuic acid and oleuropein, on LDL oxidation mediated by the J774 A.1 murine macrophage-like cells, which express CD14 and are considered to be a useful model system for the study of LDL oxidation processes (21). We examined the effects of phenolic compounds on time-dependent variations in intracellular redox balance during the oxidative process by evaluating modifications in the production of reactive oxygen species (ROS), in GSH content and related enzyme activities and/or gene expression (GPx, GR, and γ-GCS). We also assessed whether the antioxidants added to the cells have any direct effect on the modulation of intracellular redox balance, and on activity and gene expression of GSH-related enzymes.

MATERIALS AND METHODS

Materials. Protocatechuic acid was purchased from Fluka. Oleuropein was purchased from Extrasinthese SA. 2-Thiobarbituric acid, tetrametoxipropane, EDTA, and $CuSO_4$ were from Sigma Chemical. Agarose gels (Paragon LipoGel) were purchased from Beckman Coulter. RPMI 1640 culture medium, DMEM phenol red–free culture medium, fetal calf serum (FCS), Ultroser G, L-glutamine, penicillin, streptomycin, and trypsin were purchased from GIBCO BRL (Paisley, Scotland). Dihydroetidium (DHE), dihydrorhodamine 123 (DHR 123) and 5-chloromethyl-2′,7′-dichloro-dihydrofluoresceindiacetate (CM-H2-DCFDA) were from Molecular Probes. Protein content was determined by Bio-Rad Protein Assay. J774 A.1 cells were purchased from the American Tissue Culture Collection (ATCC).

Methods

Cell culture. J774 A.1 cells (5×10^5) were seeded in a 25-cm^2 flask (Falcon) and grown in RPMI 1640 medium, containing 0.2 mmol/L glutamine, 10 U/mL antibiotics and 10% FCS at 37°C, 5% CO_2.

LDL isolation. LDL were isolated from pooled fresh plasma of healthy volunteers by density gradient ultracentrifugation in a vertical rotor (Beckman Vti 50) at 180,000 × *g* for 2 h at 10°C (22). The isolated LDL were dialyzed with a centrifugal filter device (Millipore) with a molecular weight cut-off of 5000 at 4°C. Briefly, LDL were diluted 1:2 with PBS and centrifuged 5 times at 1300 × *g* for 1 h at 10°C according to the manufacturer's instructions.

LDL oxidation by J774 A.1 macrophage-like cells. One milliliter of the cell suspension at a density of 5×10^8 cells/L was added to each well of a 6-well culture plate and incubated at 37°C in 5% CO_2 for 2 h. The cells were washed twice with PBS, and 1 mL of phenol red–free DMEM containing 0.2 mmol/L glutamine, 10 kU/L antibiotics, 2% Ultroser G, and LDL (0.2 mg protein) was added to the plate and incubated at 37°C in the presence of 2 μmol/L CuSO4 for 6, 12, and 24 h. The oxidation was stopped by the addition of 1 mmol/L EDTA and 20 μmol/L BHT to prevent artifact formation of LDL oxidation. The culture medium was collected and centrifuged at 200 × *g* for 10 min to remove detached cells, and used for analytical determinations.

In the experiments that evaluated the antioxidant activity of extra virgin olive oil phenols, protocatechuic acid or oleuropein was added to the cells at different concentrations (range 1.5–25 μmol/L), following two experimental procedures. In procedure A, the biophenols were added to the cells with the LDL and left in the medium throughout the experimental period. In procedure B, the antioxidants were added to the cells for 2 h and then removed; the culture medium was renewed after washing the cells twice with PBS before the addition of LDL.

On the basis of these preliminary experiments (data not shown), the lowest concentrations able to counteract cell-mediated oxidation of LDL were determined. When protocatechuic acid or oleuropein was used, concentrations of 3 and 25 μmol/L were chosen for procedures A and B, respectively. In all experiments, untreated cells were used as a control; as further controls, LDL were incubated in the absence of cells (cell-free LDL) and J774 A.1 cells were incubated with biophenols alone. Experiments were replicated 4 times for each protocol. Each experimental treatment was performed in duplicate for each replication. The duplicates were averaged and this mean was used in the statistical analyses.

TBARS concentration. The extent of lipid peroxidation was determined by measuring the formation of TBARS in the culture medium containing LDL, according to the method of Niehaus and Samuelsson (23). Results were expressed as nmol/mg LDL protein.

Relative electrophoretic mobility (REM). Changes in electrophoretic mobility due to the oxidative modification of LDL were evaluated on 0.5% agarose gel (LipoGel Paragon, Beckman). The agarose gel was electrophoresed in barbital buffer, pH 8.6 (0.05 ionic strength) at 90 V, 40 mA, for 90 min. The electrophoretic mobility was expressed as the ratio between the distance of migration of cell-incubated LDL and that of native LDL. The assay was performed on culture medium containing LDL and on cell-free LDL resuspended in the same medium.

Protein concentration. Protein concentrations in the final LDL and in cell culture preparations were determined with Bradford's method using bovine serum albumin (BSA) as the standard (24).

Intracellular ROS. Cells (5×10^5) were incubated in 490 μL of HBSS, pH 7.4, with 5 μL of DHE or DHR123 in polypropylene test tubes for 15 min at 37°C as previously described (25). To exclude propidium iodide (PI)-positive cells from the analysis of H_2O_2 production, we performed a double-staining procedure with DHR123/PI; to estimate the percentage of dead cells in samples stained with DHE (which emits in FL2 channel like PI), we incubated parallel tubes with PI for 15 min at 37°C.

Total intracellular GSH levels. Intracellular GSH content was evaluated using CM-H_2DCFDA. Untreated and treated cells (5×10^5) were incubated with 10 μmol/L of CM-H_2DCFDA for 15 min at 37°C in HBSS supplemented with 5 g/L BSA. Cells exposed to 7.5 μmol/L L-buthionine-[*S*,*R*]-sulfoximine (Sigma), a GSH depleting drug, for 16 h were considered as negative controls (25). PI-positive cells were excluded from our analysis.

Glutathione reductase and glutathione peroxidase activities. Enzymatic activities of GR and GPx were measured by colorimetric assays, based on the oxidation and reduction of NADPH/NADP (GR-340, GPx-340, Oxis International). Samples were analyzed on a Beckman 640 spectrophotometer equipped with 25°C thermostated cuvette holder, measuring the change in absorbance at 340 nm as a function of time.

Extraction of total RNA and semiquantitation by RT-PCR. Total RNA was extracted by the TRIZOL isolation method (Gibco, BRL). The isolated RNA was used for RT-PCR analysis. PCR was performed using the following couples of primers: 5′-GGA CAG CCC TAC GGA GGA AC-3′ and 5′-GGC TTG GAA TGT CAC CTG GA-3′ for amplification of γGCS; 5′-ATG TGG AAC ACA GCA GTG CA-3′ and 5′-GTG CAC TTG GAA CTG ATG AG-3′ for GR; 5′-CCT CAA GTA CGT CCG ACC TG-3′ and 5′-TAG GAG TTG CCA GAC TGC TG-3′ for GPx; 5′-GGA AAG ACT GTT CCA AAA ACA GTG-3′ and 5′-GTC TTG GTG CTC TCC ACC TTC CG-3′ for amplification of cyclophilin B as a housekeeping gene. The samples were incubated in an automated heat-block (Minicycler, MJ Research) using the following parameters: 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min (using 31 cycles) for GR and GPx amplification; 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min for γGCS (using 35 cycles). The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Densitometric analysis was performed by a molecular imager FX (Bio-Rad).

Statistical analysis. The results are expressed as means ± SEM of at least 4 experiments performed in duplicate. Comparisons between 2 groups were by Student's *t* test. ANOVA and the Student-Newman-Keuls multiple comparison test were used when >2 groups were compared. Differences were considered significant when $P < 0.05$.

RESULTS

LDL oxidation

TBARS production in culture media. J774 A.1 significantly increased the production of TBARS in the medium

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compared with the cell-free LDL at 12 and 24 h (**Table 1**). When protocatechuic acid or oleuropein was added with LDL, or when they were added for 2 h and then removed from the medium before LDL addition, cell-mediated oxidation of the lipoproteins was completely inhibited as shown by the lack of increase in TBARS content at 24 h (Table 1).

Relative electrophoretic mobility of LDL. The relative electrophoretic mobility (REM) was expressed as the ratio between the migration distance of oxidized LDL and that of native LDL. The 24-h incubation of LDL with the macrophage cells increased the electrophoretic mobility of the lipoprotein 1.5-fold compared with native LDL (cell-free control). The extent of migration did not differ for native LDL in the presence of protocatechuic acid or oleuropein (procedure A), or when the phenolic compounds were removed before the addition of LDL (procedure B); in both the procedures, the increase in REM induced by J774 A.1 cells was fully prevented (Table 1).

Redox imbalance

Intracellular redox balance. During the oxidation of LDL, an increase in $O_2^{\cdot -}$ and H_2O_2 production and a decrease in GSH content were observed in the J774 A.1 cells. After 6 h of incubation, $O_2^{\cdot -}$ and H_2O_2 (**Fig. 1**, *A* and *B* respectively) were already increased ($P < 0.001$); GSH content (Fig. 1*C*) did not change within 6 h, but from h 12 on, it decreased progressively, reaching the lowest values at h 24, probably as a consequence of its detoxifying activity in response to ROS overproduction. Protocatechuic acid and oleuropein completely counteracted these adverse effects at every time point tested in both procedures.

Enzyme activity and mRNA expression. In the absence of extra virgin olive oil biophenols, after 24 h of incubation of cells with LDL, which infers the presence of oxLDL-mediated oxidative stress, the activity of GR was reduced compared with the control cells ($P < 0.01$). In both experimental procedures, cells treated with LDL and the biophenols had GR activity that was higher than that of LDL alone and comparable to that of control cells (**Fig. 2***A*).

Cells incubated with LDL had significantly decreased GPx activity compared with the control cells and cells treated with the biophenols (procedures A and B). It is worth noting that the cells of procedure B (Fig. 2*B*) had increased GPx activity ($P < 0.05$) compared with controls.

The expression of the enzyme γGCS gene in J774 A.1 cells incubated with LDL alone was ~100% greater at 24 h than in control cells and cells treated with the biophenols in both procedures (**Fig. 3***A*).

The expression of GR mRNA likely was affected by the LDL-mediated oxidative stress and by the consequent decrease in enzyme activity. In the cells incubated with LDL, we observed a time-dependent increase (data not shown) that was significant at 24 h compared with the control cells ($P < 0.001$) and the cells treated with LDL and biophenols ($P < 0.05$) (Fig. 3*B*).

The mRNA content for GPx was not influenced by LDL or biophenols in procedure A. Conversely, when the cells were incubated with biophenols 2 h before the addition of LDL (procedure B), protocatechuic acid and oleuropein were increased significantly compared with the control ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 3*C*).

As described in the Materials and Methods, all of the experiments were performed including cells exposed to the biophenols alone for up to 24 h to determine their possible direct influence. The biophenols influenced the variables tested similarly for both procedures, although the strongest effects were observed when they were added for 2 h and then removed from the culture medium. In particular, we observed that protocatechuic acid and oleuropein did not have any affect on ROS production or GSH content compared with control cells (data not shown), whereas they did significantly increase GR and GPx gene expression within 2 h and the related activities within 6 h after treatment with biophenols (**Fig. 4***A* and *B*). γGCS expression did not differ from that of controls (data not shown).

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TABLE 1

TBARS production and relative electrophoresis mobility (REM) during cell-mediated oxidation of LDL in J774 A.1 cells alone (LDL) or in the presence of protocatechuic acid (LDL + proto) or oleuropein (LDL + oleu), following procedure A or procedure B[1]

	TBARS		REM[2]	
	Cell-free LDL	LDL + cells	Cell-free LDL	LDL + cells
	nmol MDA/mg LDL protein			
Time-dependent LDL oxidation				
Time, *h*				
6	3.7 + 1.6	4.2 + 2.1	1.0 + 0.1	1.0 + 0.1
12	3.6 + 1.4	21.1 + 8.6#	1.0 + 0.1	2.2 + 0.3#
24	3.8 + 1.7	64.0 + 10.4^	1.1 + 0.2	2.6 + 0.3#
Biophenol effects at 24 h				
Cell-free LDL	3.8 + 1.7		1.1 + 0.2	
LDL	64.0 + 10.4*		2.6 + 0.3*	
LDL + proto (procedure A)	3.1 + 1.5		1.0 + 0.1	
LDL + proto (procedure B)	3.5 + 1.6		0.9 + 0.2	
LDL + oleu (procedure A)	4.0 + 1.7		1.2 + 0.2	
LDL + oleu (procedure B)	4.2 + 1.9		0.9 + 0.1	

[1] Values are means ± SEM, $n = 4$. #,^ Different from cell-free LDL at that time, $P < 0.05$ and 0.01, respectively. * Different from all other means in the column, $P < 0.05$.

[2] Expressed as the ratio between the distance of migration of cell-incubated LDL and that of native LDL.

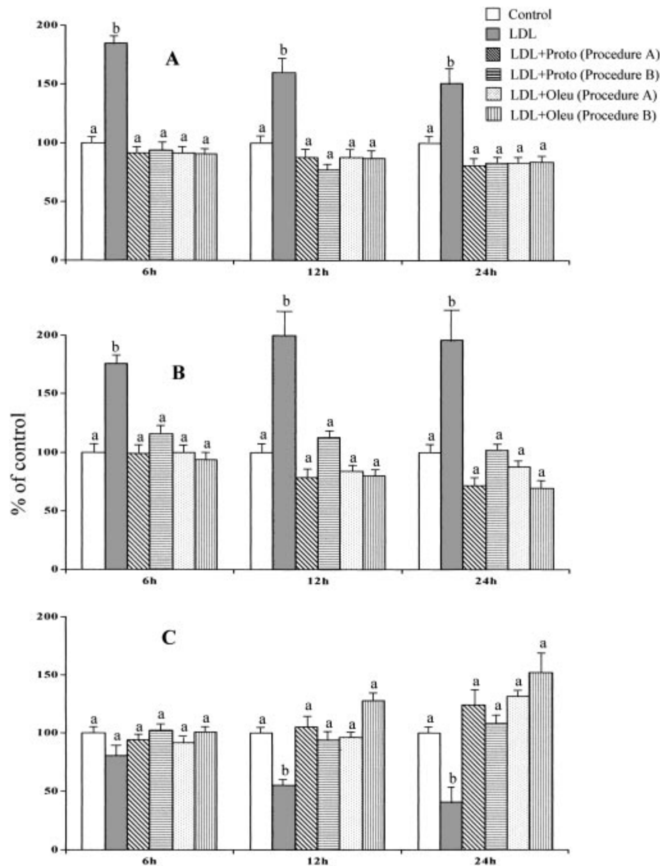

FIGURE 1 Protocatechuic acid and oleuropein counteract redox imbalance in J774 A.1 cells alone (LDL) or in the presence of protocatechuic acid (LDL + proto) or oleuropein (LDL + oleu), following procedure A or procedure B. Cytofluorimetric analysis of superoxide anion production (*A*), hydrogen peroxide production (*B*), and intracellular reduced glutathione (*C*) after 6, 12 and 24 h incubation of LDL (0.2 g protein/ L) Values are means ± SEM, $n = 4$, of the median values of the fluorescence intensity histograms after conversion of logarithmically amplified signals into values on a linear scale. Values obtained in treated cells were compared with values of control untreated cells (100%). Bars without a common letter differ, $P < 0.01$.

DISCUSSION

The lipid peroxidation hypothesis of atherosclerosis is supported by evidence of oxidized lipoproteins in atherosclerotic plaques, by the greater oxidizability of LDL isolated from patients with cardiovascular diseases, and by the protective effects exerted by some antioxidants against atherosclerosis (1).

LDL oxidation is thought to occur in the arterial wall, and it was demonstrated that macrophages, as well as endothelial cells and smooth muscle cells, oxidize LDL in vitro in the presence of catalytic amounts of transition metal ions. Some lines of evidence have also suggested that oxLDL can in turn exert powerful cytotoxic effects on macrophages, thus contributing to the development of cell-poor, lipid-rich cores of advanced atherosclerotic plaques (26). Several phenolic antioxidative compounds contained in extra virgin olive oil were shown to inhibit the LDL oxidation induced by cupric ions in vitro (27). It was demonstrated that LDL isolated from rabbits fed a standard diet modified by the addition of specific extra virgin olive oil phenolic compounds had an increased resistance to oxidation (28,29).

Although the metabolism, bioavailability, and clinical relevance of the different classes of biophenol compounds are subject to debate, phenolic compounds of extra virgin olive oil are absorbed and, as reported (30), bind to plasma lipoproteins after consumption. Moreover, a previous study suggested that the daily consumption of extra virgin olive oil increased the phenolic compounds in plasma and the resistance of LDL against ex vivo oxidation in hyperlipidemic patients (31). The maximum concentration of protocatechuic acid and oleuropein in extra virgin olive oil was estimated to be 19.5 and 5.6 μmol/kg, respectively (32); thus, if we assume a daily consumption of ~50 g of olive oil (33), the intake was very low. However, we should also take into account the following: *1*) oleuropein normally associates with its derivatives hydroxytyrosol and aglicone (reaching total concentrations of 125 μmol/(kg · d) (32); and *2*) protocatechuic acid is recognized as one of the metabolites formed during the absorption of complex polyphenols (34,35). For example, proanthocyanidins and catechins are normally present at high concentrations in vegetables and fruits. This can considerably increase dietary intake.

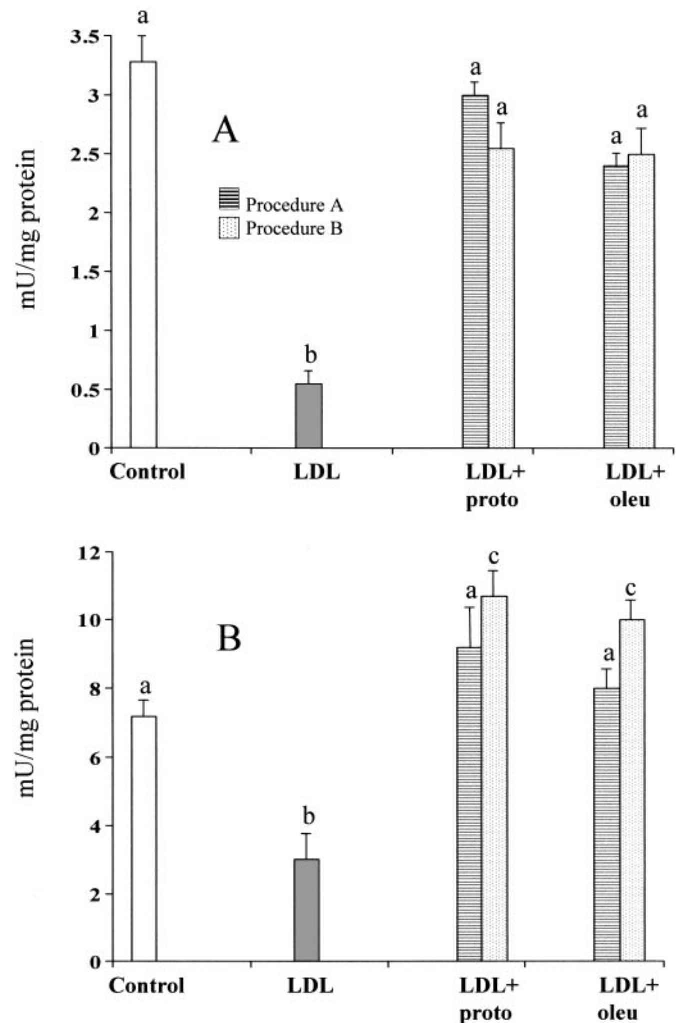

FIGURE 2 Biophenols restore GR (*A*) and GPx (*B*) activities in J774 A.1 macrophage-like cells following both procedure A or procedure B. Activities were measured after a 24-h incubation with LDL (0.2 g protein/L). Values are means ± SEM, $n = 4$. Bars without a common letter differ, $P < 0.05$. LDL = cell exposed to LDL; LDL + proto = cell exposed to LDL and protocatechuic acid; LDL + oleu = cell exposed to LDL and oleuropein.

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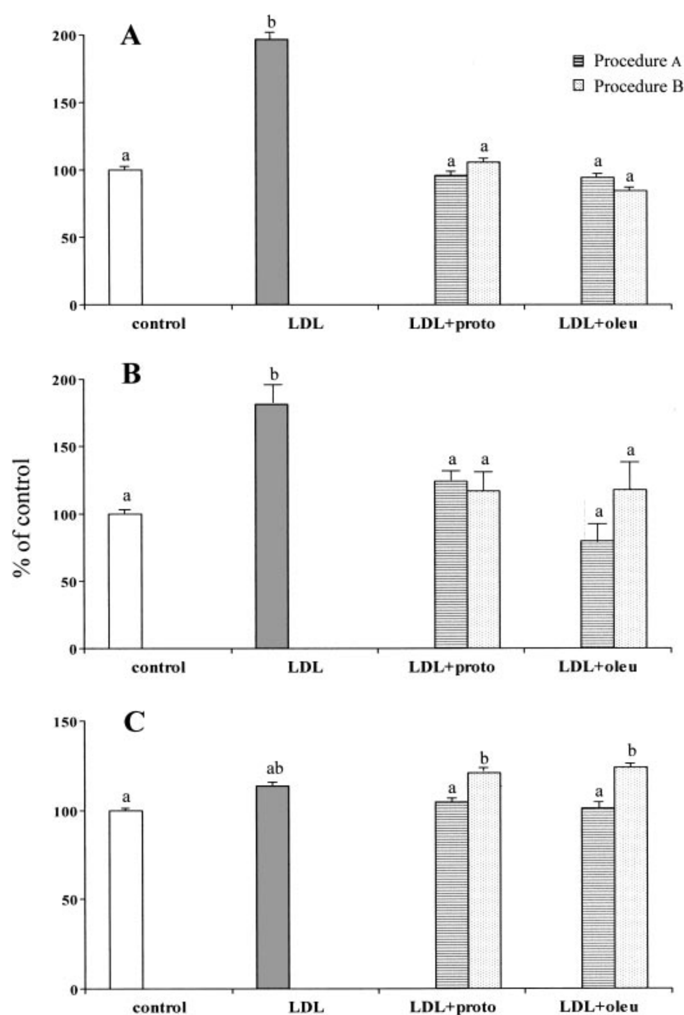

FIGURE 3 Gene expressions for γGCS, GR and GPx in J774 A.1 cells after a 24-h incubation with LDL (0.2 g protein/L) alone and with protocatechuic acid or oleuropein following procedure A or procedure B. Semiquantitative RT-PCR evaluation of mRNA production for: γGCS **(***A***)**, GR **(***B***)** and GPx **(***C***)** genes. Values are means ± SEM, n = 4. Bars without a common letter differ, $P < 0.05$. LDL = cell exposed to LDL; LDL + proto = cell exposed to LDL and protocatechuic acid; LDL + oleu = cell exposed to LDL and oleuropein.

Absorbed compounds could be taken up by the cells, including macrophages, and affect the cellular oxidative status (36).

In the present study, we demonstrated that the phenolic compounds contained in extra virgin olive oil, namely, protocatechuic acid and oleuropein, as previously demonstrated for some polyphenols of red wine (37), inhibit macrophage-mediated oxidation of LDL. Importantly, we provide evidence that the mechanisms underlying this specific activity of biophenols are associated with their "boost" effect on intracellular antioxidizing properties. In fact, these biophenols, together with LDL in the culture medium, counteracted TBARS production and the increase in electrophoretic mobility associated with the oxidation of lipoproteins, thus preventing the peroxidation of their lipid components. These findings could be ascribed to the ability of protocatechuic acid and oleuropein to act as scavengers for radicals and chain-breaking antioxidants, both actions that are related to the *ortho-* and *para*-hydroxyl groups present on the phenol ring. On the other hand, lipid peroxidation inhibition seems to depend on the intracellular accumulation of these biophenols and on their capability to in some way block some of the early intracellular events necessary to begin the oxidative process in lipoproteins. In fact, it is worth noting that, when incubated with the macrophage-like J774 A.1 cells for 2 h and then removed from the culture medium before LDL addition, protocatechuic acid and oleuropein exerted a potent inhibitory effect on the macrophage-mediated oxidation of LDL.

LDL oxidation mediated by macrophage cells depends on the intracellular balance between prooxidants and antioxidants, including the glutathione system, (4,6) and involves the activation of NADPH oxidase (38), which produces superoxide anions that are able to convert native LDL to atherogenic oxLDL. Redox imbalance in J774 A.1 cells was already evident after 6 h of incubation with LDL and a catalytic amount of copper, from the increase in ROS production and, after 12 h, from the decrease in GSH content with respect to the control cells. Extra virgin olive oil biophenols inhibited the redox imbalance by scavenging released radicals, thus preserving the intracellular GSH content; they also downregulated the production of superoxide anions, as shown when the biophenols were present before LDL addition and then removed from the medium. These data are in agreement with those recently obtained by Léger et al. (39) in cultured human monocyte THP-1 cells treated with mixtures of wastewaters from olive oil processing. Some questions remain about the effect that the biophenols tested here have on cell activity, e.g., effects on the assembling of the NADPH oxidase complex and on the activation of protein kinase C, shown for other antioxidants (40–42).

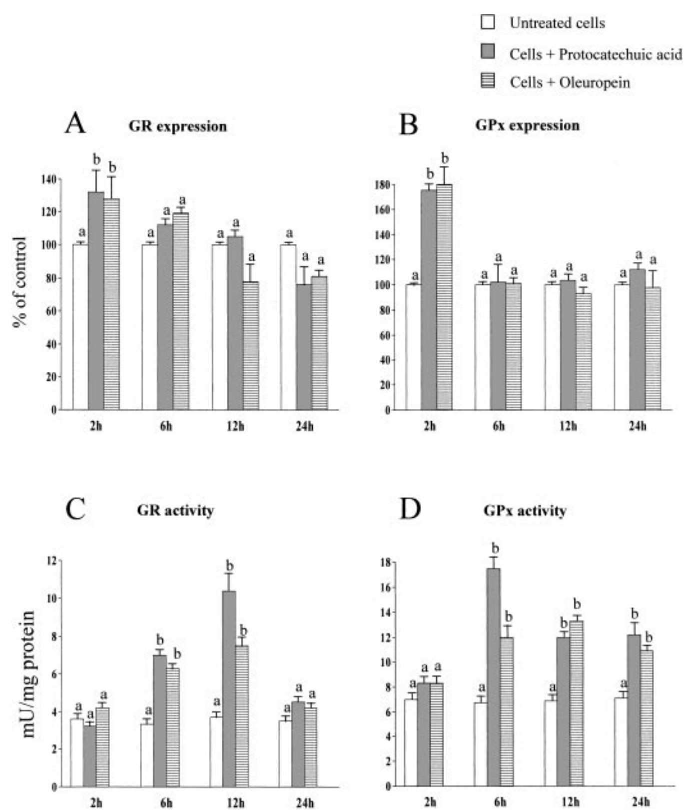

FIGURE 4 Direct effect of the biophenols on DNA transcription of GSH-related enzymes in J774 A.1 cells incubated with protocatechuic acid and oleuropein following procedure B. (*A*) Semiquantitative RT-PCR time-course evaluation of mRNA for GR and GPx. (*B*) Time-course evaluation of GR and GPx activities. Values are means ± SEM, n = 4. Bars without a common letter differ, $P < 0.05$.

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Another possible key mechanism in the protective effect exerted by extra virgin olive oil biophenols could involve the sparing of GSH, i.e., the strengthening of intracellular antioxidant defenses. It was demonstrated that macrophage-mediated oxidation of LDL and cellular lipid peroxidation increase in glutathione-depleted cells (4). Reduced glutathione is a critical factor in the cellular antioxidant network as are its related enzymes GPx, which catalyzes the elimination of a variety of hydroperoxides including H_2O_2 and organic hydroperoxides, and GR, which regenerates GSH from oxidized glutathione (43,44). The functional efficiency of this redox cycle in peroxide catabolism will depend on the optimal activities of the redox enzymes, GPx and GR, and the optimal supply of the reductant GSH. An extensive depletion of the GSH pool will prevent further peroxide degradation, thus compromising tissue cell integrity and representing a stimulus for its de novo synthesis by two sequential ATP-dependent reactions catalyzed by γGCS, the rate-limiting enzyme, and by glutathione synthetase (45).

The data we report here suggest that one of the mechanisms by which extra virgin olive oil biophenols inhibited cell-mediated oxidation of LDL was by preventing ROS accumulation through the improvement of the entire GSH redox cycle, as demonstrated for other antioxidative compounds (46). The preservation of enzyme efficiency, and consequently of the normal content of GSH, is also confirmed by the lack of stimulation of mRNA for γGCS in the cells treated with biophenols and LDL, compared with the cells treated with LDL alone in which the depletion of GSH was a potent stimulus for γGCS gene expression (47). The observed up-regulation may indicate a reaction of the cells to meet the increasing demand of glutathione.

The most interesting results were the specific actions exerted by protocatechuic acid and oleuropein on the expression and activity of glutathione peroxidase and glutathione reductase. We demonstrated an increase in mRNA overproduction and enzyme activity in cells pretreated with protocatechuic acid and oleuropein, with and without LDL compared with controls.

It is noteworthy that the two experimental procedures allowed us to distinguish different mechanisms through which the biophenols exerted their protective effects. Following procedure A, we demonstrated their classical antioxidative action as radical scavengers or chain-breakers of peroxidative reaction; with procedure B, we showed an additional intracellular mechanism through the direct activation of mRNA transcription of the detoxifying enzymes. These findings were also confirmed by the potentiated enzyme activity in cells treated with the biophenols alone. The use of in vitro systems can help clarify the mechanisms underlying the strengthening of the cellular defenses exerted by the phenols. Further studies are warranted, however, before these agents can be considered in clinical practice.

We conclude that the protective effect exerted by biophenols may involve more than one mechanism. The observed increase in mRNAs, with no oxLDL-induced alteration (Fig. 4), indicates a direct effect of the biophenols on the DNA transcription of GSH-related enzymes, and suggests that this is one of the mechanisms that improves antioxidative cellular defenses.

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