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Apoptosis induced by oxidized lipids is associated with up-regulation of p66Shc in intestinal Caco-2 cells: protective effects of phenolic compounds

Claudio Giovannini[a,*], Beatrice Scazzocchio[a], Paola Matarrese[b], Rosaria Varì[a], Massimo D'Archivio[a], Roberta Di Benedetto[a], Stefania Casciani[c], Maria Rita Dessì[c], Elisabetta Straface[b], Walter Malorni[b,1], Roberta Masella[a,1]

[a]*National Centre for Food Quality and Risk Assessment, Istituto Superiore di Sanità, 299-00161 Rome, Italy*
[b]*Department of Drug Research and Evaluation, Istituto Superiore di Sanità, 299-00161 Rome, Italy*
[c]*Department of Internal Medicine, II University of Rome "Tor Vergata", 1-00133 Rome, Italy*

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Abstract

In this study, we investigated the alterations of the redox balance induced by the lipid fraction of oxLDL in Caco-2 intestinal cells, and the effects of tyrosol and protocatechuic acid, two dietary phenolic compounds. We found that oxidized lipids extracted from oxLDL (LipE) induced oxidative stress by determining, 6 h after treatment, ROS overproduction (about a 100% and a 43% increase of O_2^- and H_2O_2 production, respectively, $P<.05$: LipE vs. control) and, 12 h after treatment, GSH depletion (about a 26% decrease, $P<.05$: LipE vs. control), and by impairing the activities of superoxide dismutase, catalase and glutathione peroxidase. In response to the induced oxidative stress, we observed significant overexpression of glutathione peroxidase (6 h after treatment: $P<.05$), glutathione reductase and γ-glutamylcysteine synthetase (12 h after treatment: $P<.05$). Notably, when GSH depletion occurred, p66Shc protein expression increased by about 300% with respect to control ($P<.001$; LipE vs. control). These effects were fully counteracted by dietary phenolics which inhibited ROS overproduction and GSH consumption, rendered the reactive transcription of glutathione-associated enzymes unnecessary and blocked the intracellular signals leading to the overexpression and rearrangement of p66Shc signalling molecule. Altogether, these results suggest that the impairment of the antioxidant system hijacks intestinal cells towards an apoptotic-prone phenotype via the activation of p66Shc molecule. They also propose a reappraisal of dietary polyphenols as intestinal protecting agents, indicating the antiapoptotic effect as a further mechanism of action of these antioxidant compounds.
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Keywords: Apoptosis; oxLDL; Oxidized lipids; Caco-2; Glutathione; p66Shc; Polyphenols

1. Introduction

In the gastrointestinal tract, prooxidants such as endogenous and exogenous lipid peroxides have been demonstrated to impact intestinal integrity. In fact, cellular and molecular events involved in degenerative pathological processes leading to intestinal disorders have been associated with redox alterations. Being the interface between the organism and its luminal environment, the intestine is constantly challenged by diet-derived oxidants as well as by endogenously generated reactive species or oxidants. In particular, a high intake of dietary polyunsaturated fatty acids can contribute to the luminal accumulation of lipid hydroperoxides, and the subsequent lipid peroxidation can induce oxidative stress and redox imbalance, contributing to the development of gut pathologies, such as inflammation and cancer [1,2]. To preserve cellular integrity and tissue homeostasis, the intestine possesses several defence

Abbreviations: CAT, catalase; DHE, dihydroethidium; DHR 123, dihydrorhodamine 123; GPx, glutathione peroxidase; GSSGred, glutathione reductase; γ-GCS, γ-glutamylcysteine synthetase; GSH, glutathione; LipE, lipid extract from oxLDL; MBC, monochlorobimane; oxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; SOD, superoxide-dismutase.

* Corresponding author. Tel.: +39 06 49902589; fax: +39 06 49902763.
E-mail address: clagiovn@iss.it (C. Giovannini).

[1] Both should be considered as senior author.

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mechanisms such as the ability to maintain high antioxidant levels (glutathione, tocopherol and ascorbic acid), to up-regulate the antioxidant enzyme system (glutathione peroxidase, glutathione reductase and superoxide dismutase) and to induce cell death by apoptosis in order to dispose of injured or spent cells.

There is an increasing interest in the mechanisms of response of the intestinal epithelium to oxidative stress and in the capability of nutritional antioxidants to strengthen endogenous antioxidant defences [3,4]. Among diet antioxidants, polyphenols, naturally occurring in vegetables, fruits and plant-derived beverages such as tea, red wine and extra virgin olive oil, are the most abundant ones. They can contribute to the prevention of several oxidative stress-associated diseases, characterized by inflammatory injuries, including injury of the intestine [5–8]. Polyphenols exert their protective action as reducing agents, but increasing evidence exists that they may improve antioxidant defences through the induction of antioxidant and phase II enzymes [9,10]. Moreover, several other protective effects have been recently described such as antiviral, antimicrobial, anti-inflammatory and anticarcinogenic effects, as well as the ability to interact with cell receptors or to modulate certain signalling pathways [11].

Low-density lipoproteins undergoing oxidative modification (oxLDL) generate a mixture of compounds with cytotoxic activity, i.e., lipid hydroperoxides, aldehydes and oxysterols. OxLDL with their oxidized lipid component could thus represent a suitable “physiological” model to study the effects of oxidized lipids in inducing intracellular oxidative stress. It is well known that the interaction of oxLDL oxidant products with cells and tissues results in oxidative imbalance and leads to cell death by apoptosis [12,13], but the mechanism is still unclear. We have previously demonstrated that oxLDL induces apoptosis in intestinal Caco-2 cells via the intrinsic pathway, i.e., the mitochondrial pathway [14,15]. Since the Caco-2 cell line retains many of the morphological and enzymatic features typical of normal human enterocytes [16], it is largely used as a model system for evaluating the effects of normal dietary constituents as well as additives, contaminants, toxicants, oxidants and drugs [17,18].

The aim of the present work was to investigate (i) the redox imbalance underlying apoptosis induced by oxidized lipids in Caco-2 cells in terms of expression and/or activity of the main actors of cell homeostasis, i.e., reduced glutathione (GSH), superoxide-dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSSGred) and γ-glutamylcysteine synthetase (γ-GCS); (ii) the ability of two extra virgin olive oil phenolic compounds, namely, tyrosol and protocatechuic acid, to counteract the pro-oxidant subcellular effects of oxidized lipids; and (iii) the intracellular behavior — i.e., expression and localization — of p66Shc, an oxidative stress sensor protein recently identified as an important cytoplasmic signal transducer that regulates the apoptotic response to oxidative stress [19].

2. Materials and methods

2.1. LDL isolation and oxidation

LDL (1.019–1.063 g/ml) was prepared from freshly isolated pooled plasma from healthy human donors by density gradient ultracentrifugation and then oxidized with 5 μM $CuSO_4$ for 18 h at 37°C as reported elsewhere [20].

2.2. Lipid extraction from native and oxidized LDL

Lipids were extracted from native LDL (nLDL) and oxLDL with chloroform/methanol mixture (2:1 vol/vol) containing 5 μg/ml butylated hydroxytoluene [21]. The organic phase was dried under nitrogen and the content of extracts was determined by microgravimetry. The lipid residue was dissolved in ethanol and added to culture medium (0.2 mg LDL protein equivalent/ml culture medium).

2.3. Caco-2 cell culture and experimental procedure and treatments

Caco-2 cells (European Collection of Cell Culture, Salisbury, UK) were cultured as previously reported [14]. On culture Day 5, when cells are at the initial step of their differentiation process, the medium with serum was replaced by DMEM containing 2% Ultroser G (a lipoprotein-free serum substitute). Then the cells were exposed to 0.2 mg protein/ml of nLDL or oxLDL with and without phenolic compounds. All the experiments performed at 6, 12, 18, 24 and 48 h after exposure to oxLDL or lipid extract from oxLDL (LipE) included, as controls, (i) untreated cells, (ii) cells treated with nLDL and (iii) cells treated with the lipid extract from nLDL (nLipE). Because the results obtained in oxLDL-treated cells and in LipE-exposed cells were completely overlapping, we report only the results obtained with lipid extracts. In the same vein, as controls, only the results obtained from untreated cells are shown. In the experiments to evaluate the antioxidant activity of extra virgin olive oil phenols, 0.5 mM tyrosol or 0.25 mM protocatechuic acid concentrations were chosen on the basis of preliminary experiments performed to evaluate the minimal effective concentrations. The phenolic compounds were added 2 h before oxLDL or LipE treatment and were maintained in the medium throughout the exposure to oxidized lipids.

2.4. Cytofluorimetric measurements of intracellular ROS and GSH levels

To evaluate O_2^- and H_2O_2 intracellular production, cells (5×10^5) were incubated in 490 μl of Hanks' balanced salt solution (HBSS, pH 7.4) with 1 μM dihydroethidium (DHE, Molecular Probes) or 10 μM dihydrorhodamine 123 (DHR 123, Molecular Probes), respectively, in polypropylene test tubes for 5 min at 37°C [22]. Because fluorescent oxidation products are produced in metabolically active cells only, both DHR 123 and DHE can also be used as viability indicators. Intracellular GSH content was assessed by using monochlorobimane (MBC, Molecular Probes) as previously

described by Sahaf et al. [23]. Briefly, control and treated Caco-2 cells (5×10^6) were stained with 40 μM MBC at room temperature for 20 min. As methodological control, to decrease GSH by inhibiting GSH synthesis, Caco-2 cells were incubated for 24 h with 250 mM buthionine sulfoximine (BSO, Sigma). Samples were washed twice in ice-cold PBS and immediately acquired by an LRS II cytometer (Becton and Dickinson, San Jose, CA, USA) equipped with a 488 argon laser and a UVB laser.

2.5. Spectrophotometric determination of reduced and oxidized glutathione

The level of GSH and the GSH/GSSG ratio were determined by the Bioxytech GSH/GSSG-412 assay kit (OXIS International, Inc., Portland, OR, USA) based on the spectrophotometric determination of a detectable product at 412 nm, formed by the reaction of the reagent with GSH. GSSG was determined by the same reaction after the reduction to GSH.

2.6. Measurement of glutathione reductase, glutathione peroxidase, catalase and superoxide dismutase activities

The enzymatic activities were determined by colorimetric assays for GSSGred (Bioxytech GR-340; OXIS) and GPx (Bioxytech GPx-340; OXIS) based on the oxidation of NADPH. Catalase activity was assayed by the Cayman Catalase assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically by the change in color of the chromogen when it bounds to aldehydes. Superoxide dismutase activity was measured according to Marklund and Marklund [24]. The decrease in absorbance at 340 nm for GPx and GSSGred, and at 420 nm for SOD, was measured as a function of time on a Beckman 640 spectrophotometer equipped with a temperature-regulated cuvette holder at 37°C. The increase in absorbance for CAT was monitored at 540 nm using a plate reader.

2.7. RNA semiquantitation by reverse transcriptase–polymerase chain reaction

Total RNA from cultured Caco-2 was extracted by the TRIZOL isolation method (Gibco, BRL). One microgram of total RNA was used for reverse transcription polymerase chain reaction (RT-PCR) analysis. PCR was performed using the following couples of primers: 5′-GTAATGGACCAGTGAAGGTGT-3′ and 5′-CAATTACACCACAAGCCAAACG-3′ (Ta=60°C) for SOD; 5′-GGACAGCCCTACGGAGGAAC-3′ and 5′-GGCTTGGAATGTCACCTGGA-3′ (Ta=65°C) for γGCS; 5′-ATGTGGAACACAGCAGTGCA-3′ and 5′-GTGCACTTGGAACTGATGAG-3′ (Ta=58°C) for GSSGred; 5′-CCTCAAGTACGTCCGACCTG-3′ and 5′-TAGGAGTTGCCAGACTGCTG-3′ (Ta=65°C) for glutathione peroxidase (GPx). GAPDH was used as housekeeping gene. The samples were incubated in an automated heat block (Minicycler, MJ Research). The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Densitometric analysis was performed by a molecular imager FX (Biorad Laboratories, Inc., Hercules, CA, USA).

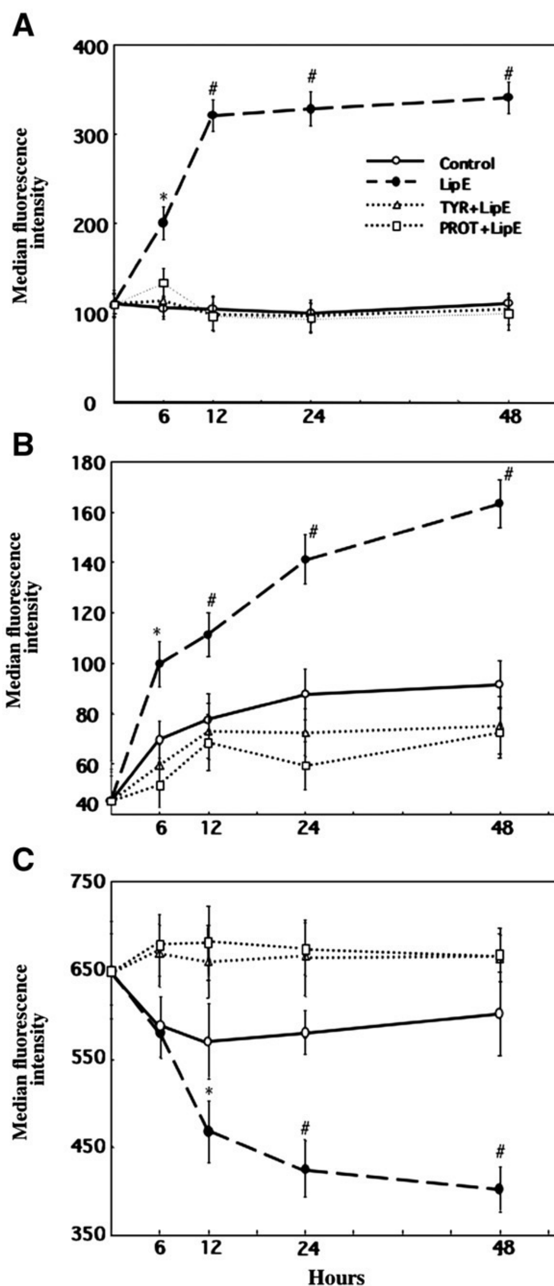

Fig. 1. Oxidized lipids induced redox imbalance in intestinal cells. Cytofluorimetric analysis of (A) hydrogen peroxide production, (B) superoxide anion production and (C) intracellular reduced glutathione, after 6, 12, 24 and 48 h of exposure to LipE, with or without the presence of tyrosol (TYR+LipE) or protocatechuic acid (PROT+LipE). Values reported are the mean of four independent experiments±S.E.M. of the median values of the fluorescence intensity histograms. *P<.05, #P<.001: LipE vs. control; LipE vs. TYR+LipE; LipE vs. PROT+LipE for each parameter and time point considered.

2.8. Quantitative and qualitative analyses of p66Shc protein

Evaluation of p66Shc was carried out by static and flow cytometry. Control and treated cells were fixed and permeabilized with cold acetone–methanol 1:1 (v/v) for 10 min. For double staining immunofluorescence analyses, cells were incubated with monoclonal antibodies against mitochondria (Chemicon International Inc. Temekula, CA, USA) or SelectFX Alexa Fluor 488 Endoplasmic Reticulum Labeling Kit (Molecular Probes), which recognizes the ER-associated protein disulfide isomerase, and polyclonal anti-p66Shc antibodies (BD Transduction Laboratories, San Jose, CA, USA). Cells were then washed with PBS and incubated with a TRITC-conjugated anti-rabbit antibody (Sigma) and FITC-conjugated anti-mouse antibody (Sigma) for 30 min at 37°C. For analytical cytology analysis, after washing, cells were mounted on glass coverslips and observed by laser scanning confocal microscopy (Leica TCS SP2, Germany), with a 63× objective. Images were collected at 512×512 pixels and processed with the Leica confocal software 4.7. For semiquantitative flow cytometry analysis of p66Shc, only a single staining with the specific antibody was performed. In this case, we used, as secondary antibody, an FITC-conjugated anti-rabbit antibody, excitable by a 488 argon laser. Samples were acquired and recorded by a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA, USA). At least 20,000 events were acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis.

2.9. Western blot analysis of p66Shc protein

For immunoblotting determination of p66Shc, nuclear protein extracts were prepared by Nuclear/Cytosol fractionation kit (Medical & Biological Laboratories, Watertown, LA, USA), according to the manufacturer's instructions. Protein concentrations were determined by the Biorad Protein Assay. Thirty micrograms of sample protein was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes, blocked with PBS containing 5% nonfat dry milk and 0.1% Tween 20, were treated with polyclonal antibody directly against p66Shc (BD Transduction Laboratories) protein. The blots were treated with appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) followed by ECL detection (Amersham Biosciences, UK). Densitometric analysis was performed with a molecular imager FX (Biorad, Hercules, CA, USA). Equal loading of proteins was verified by GAPDH immunoblotting with a goat anti-GAPDH antibody (Santa Cruz Biotechnology).

2.10. Statistical analysis

All the data are reported as the arithmetic mean from, at least, four independent experiments performed in duplicate±S.E.M. Comparisons between fluorescence histograms obtained by flow cytometry were performed by the Kolmogorov–Smirnov (K/S) test. Statistical analysis of other results reported herein was performed by using Student's *t*-test or one-way variance analysis by using the Statview program for Macintosh. Only *P* values of less than .05 were considered as statistically significant.

3. Results

3.1. Oxidized lipids induced redox imbalance in intestinal cells

Since cell death by apoptosis can be considered the final event of a redox imbalance, we carried out time course experiments to evaluate the intracellular production of ROS. The production of intracellular O_2^- increased early (100% with respect to control) after exposure to LipE (6 h) reaching a plateau within 12 h (Fig. 1A). Similarly, under the same experimental conditions, H_2O_2 production (about 43% with respect to control) was observed 6 h after oxidized lipid administration and constantly increased, reaching a plateau at 48 h (Fig. 1B).

Since glutathione is the major nonenzymatic regulator of intracellular redox homeostasis, we monitored GSH concentration during oxidative injury. GSH content monitored by flow cytometry did not change within 6 h, but, as from Hour 12 after oxidized lipid treatment (26% decrease with respect to control), it progressively decreased reaching the lowest value of about 60% depletion at Hour 48 (Fig. 1C). GSH level was further investigated by utilizing a colorimetric method that confirmed the results obtained by flow cytometry. GSH content was significantly reduced ($P<.05$) at Hour 12 after LipE exposure (Table 1).

Table 1
Glutathione redox state of Caco-2 cells during oxidized lipid treatment with or without antioxidants

	GSH (mmol/mg protein)			GSH/GSSG ratio		
	Controls	LipE	PROT+LipE	Controls	LipE	PROT+LipE
6 h	146.8±8.3	140.0±7.4	180.5±5.6	16.3±0.2	16.2±0.8	16.0±1.2
12 h	193.7±4.9	130.1±10.1*	209.8±8.8	19.2±0.8	16.4±0.3*	20.7±0.4
24 h	191.1±5.6	148.9±6.6*	230.8±11.1	19.6±0.2	17.9±0.4*	20.6±0.3
48 h	149.1±9.2	105.7±7.5*	150.3±6.2	22.6±0.3	19.8±0.4*	24.8±0.4

Data are expressed as mean±S.E.M.
LipE: cells treated with lipid extract from oxLDL; PROT+LipE: cells treated with 0.25 mM protocatechuic acid and then exposed to LipE.
* $P<.05$ vs. controls.

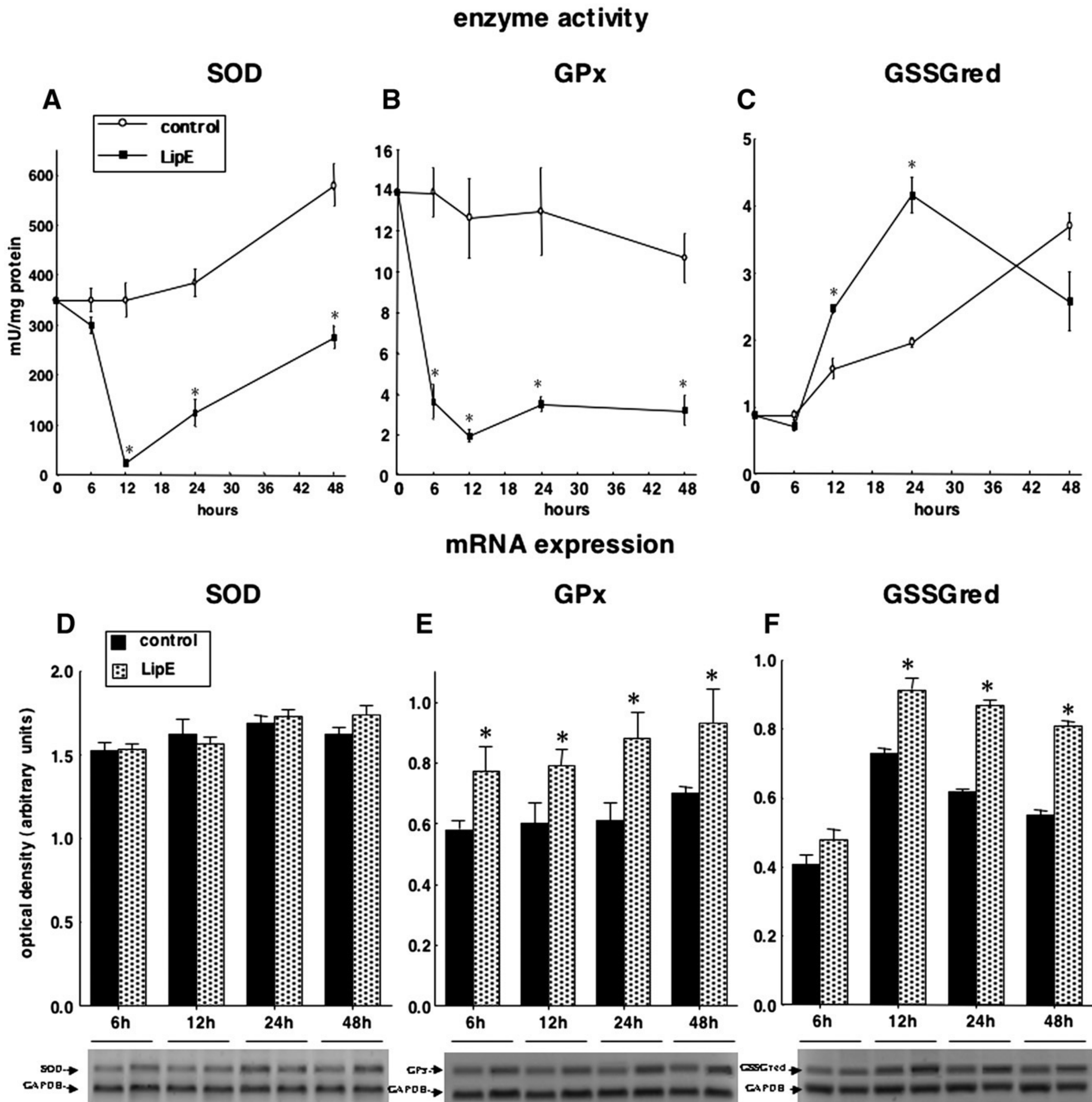

Fig. 2. Oxidized lipids affected the activity and gene transcription of SOD, GPx and GSSGred. Evaluation of (A) SOD activity, (B) GPx activity and (C) GSSGred activity by spectrophotometry. Evaluation of mRNA transcription obtained by RT-PCR for SOD (D), GPx (E) and GSSGred (F). Values reported are the mean±S.E.M. of four independent time-course experiments, observed in untreated cells (control), LipE-treated cells (LipE) and LipE-treated cells incubated with tyrosol (TYR+LipE) or protocatechuic acid (PROT+LipE) at different exposure times. Representative agarose gels obtained by RT-PCR from four independent experiments are shown for SOD (D), GPx (E) and GSSGred (F); GAPDH was used as housekeeping gene. *$P<.05$: LipE vs. control; LipE vs. TYR+LipE; oxLDL vs. PROT+LipE for each parameter and time point considered.

Because the redox potential of glutathione is a relevant parameter for the cellular redox state rather than GSH level itself, the ratio between reduced and oxidized glutathione was investigated. We found that GSH/GSSG ratio decreased from Hour 12 after LipE treatment, clearly indicating the induction of oxidative stress (Table 1).

When the cells were treated with the phenolic compounds for 2 h before oxLDL or LipE exposure, the presence of tyrosol or protocatechuic acid in the medium fully protected them against oxidized lipid-induced adverse effects. In fact, control values of hydrogen peroxide and superoxide anion production remained substantially unchanged (Fig. 1A and B), while values of the GSH content and the ratio of GSH/GSSG (Fig. 1C, Table 1) were even higher than those observed in control cells, indicating that the presence of phenolic compounds, per se, can increase the endogenous antioxidant capacity of the cell system.

3.2. Oxidized lipids modified the activity and expression of antioxidant enzymes

The presence of GSH is essential, but not by itself sufficient, to prevent the cytotoxicity of ROS. In fact, a series of antioxidant enzymes contribute to cellular anti-oxidative defences. Thus, a time-dependent analysis of these enzymes was carried out. In particular, modifications induced by LipE in the activity and/or mRNA expression of the main detoxifying enzymes, i.e., SOD, CAT, GPx, GSSGred and γ-GCS, were evaluated. We found that SOD activity was significantly impaired after 12 to 48 h of oxLDL or LipE exposure (Fig. 2A). By contrast, its mRNA synthesis remained unaffected (Fig. 2D). Early after LipE exposure (6 h), when H_2O_2 overproduction was observed, CAT activity was impaired. The enzyme activity was reduced by about 50% with respect to controls after 12 h (25.9±4.3 vs. 51.7±2.1 nmol/min per mg protein) and decreased constantly, reaching the lowest level after 48 h (8.1±1.3 vs. 37.3±2.6 nmol/min per mg protein). In the presence of oxidized lipid-mediated oxidative stress, the activity of GPx was impaired early and dramatically reduced at each time point considered (6, 12, 18, 24 and 48 h) (Fig. 2B). Moreover, the time course analysis (6–48 h) of gene expression clearly indicated a significant increase in the mRNA of this enzyme with respect to control samples ($P<.05$) at each time point considered (Fig. 2E). Interestingly, we also observed an increase in both activity and gene expression ($P<.05$) of GSSGred (Fig. 2C and F) and, noteworthily, this was detected 12 h after exposure to oxidized lipids, corresponding to the above-reported decrease in intracellular GSH. Finally, we studied the gene expression of γ-GCS, the limiting enzyme responsible for GSH de novo synthesis. We observed that treatment with oxLDL or LipE caused an early (6 h) significant up-regulation of the enzyme mRNA expression ($P<.05$) corresponding to an average increase of 64.8±3.2% with respect to control cultures.

Tyrosol or protocatechuic acid can exert their protective action on cellular antioxidant defenses by inhibiting ROS overproduction and GSH consumption, which renders the reactive transcription of glutathione-associated enzymes unnecessary. In fact, as reported in Fig. 3, cell incubation with the phenolic compounds 2 h before and throughout LipE treatment led to a significant protection against the induced redox imbalance, at least in terms of mRNA transcription of GPx, GSSGred and γ-GCS (Fig. 3A–C).

3.3. p66Shc expression and localization

To investigate the role of p66Shc in oxidized lipid-induced apoptosis, protein expression during exposure to oxidized lipids was firstly quantified by means of flow cytometry analysis. We found that p66Shc expression was significantly up-regulated after 12 h of exposure to oxidized lipids (Fig. 4A and B). Similarly, increased p66Shc protein expression was observed with Western blotting analysis throughout the exposure to oxidized lipids, with the highest levels, corresponding to an increase of about 300% with respect to controls, at Hour 12 (Fig. 4C). However, at later time points, p66Shc expression came back to basal values. Interestingly, pretreatments with phenolic antioxidants potently counteracted p66Shc up-regulation. It is worth noting that the protein overexpression (12 h) corresponded to the above-reported decrease in intracellular GSH.

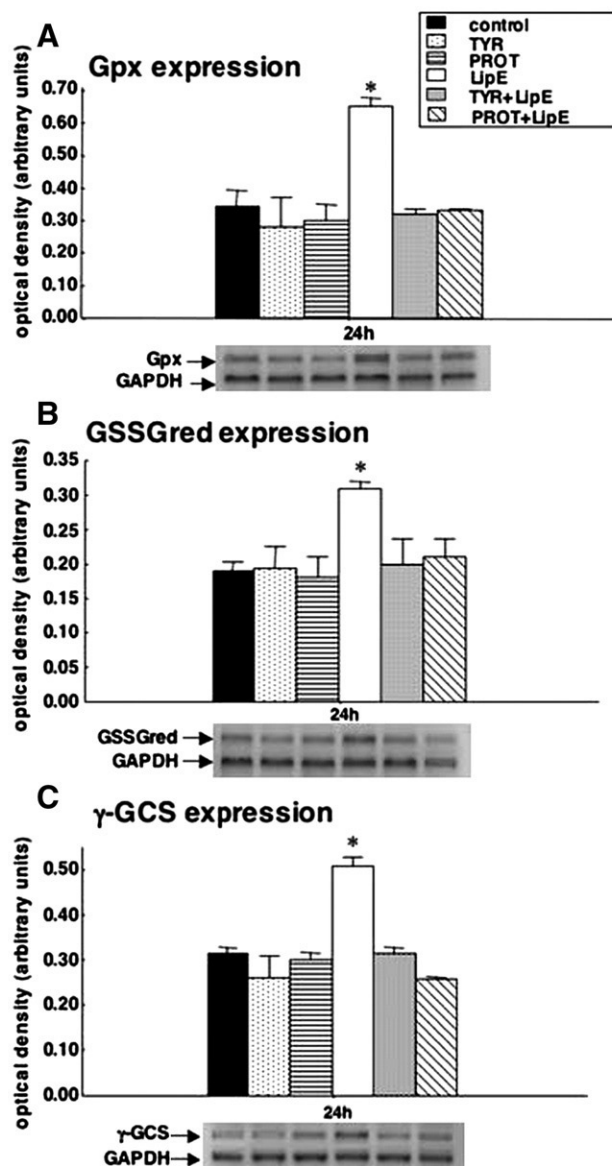

Fig. 3. Polyphenols restored mRNA expression of glutathione-related enzymes. mRNA expression of GPx (A), GSSGred (B) and γ-GCS (C) obtained by RT-PCR, reported as optical density, after 24 h of exposure to LipE (LipE), Lip E in the presence of tyrosol (TYR+LipE) or protocatechuic acid (PROT+LipE), and to tyrosol (TYR) or protocatechuic acid (PROT) alone, as further controls. Values are the mean±S.E.M. of four independent experiments, performed in duplicate (*$P<.05$ vs. control, for each parameter and time point considered). A representative agarose gel obtained by RT-PCR from four independent experiments is shown for GPx (A), GSSGred (B) and γ-GCS (C); GAPDH was used as housekeeping gene.

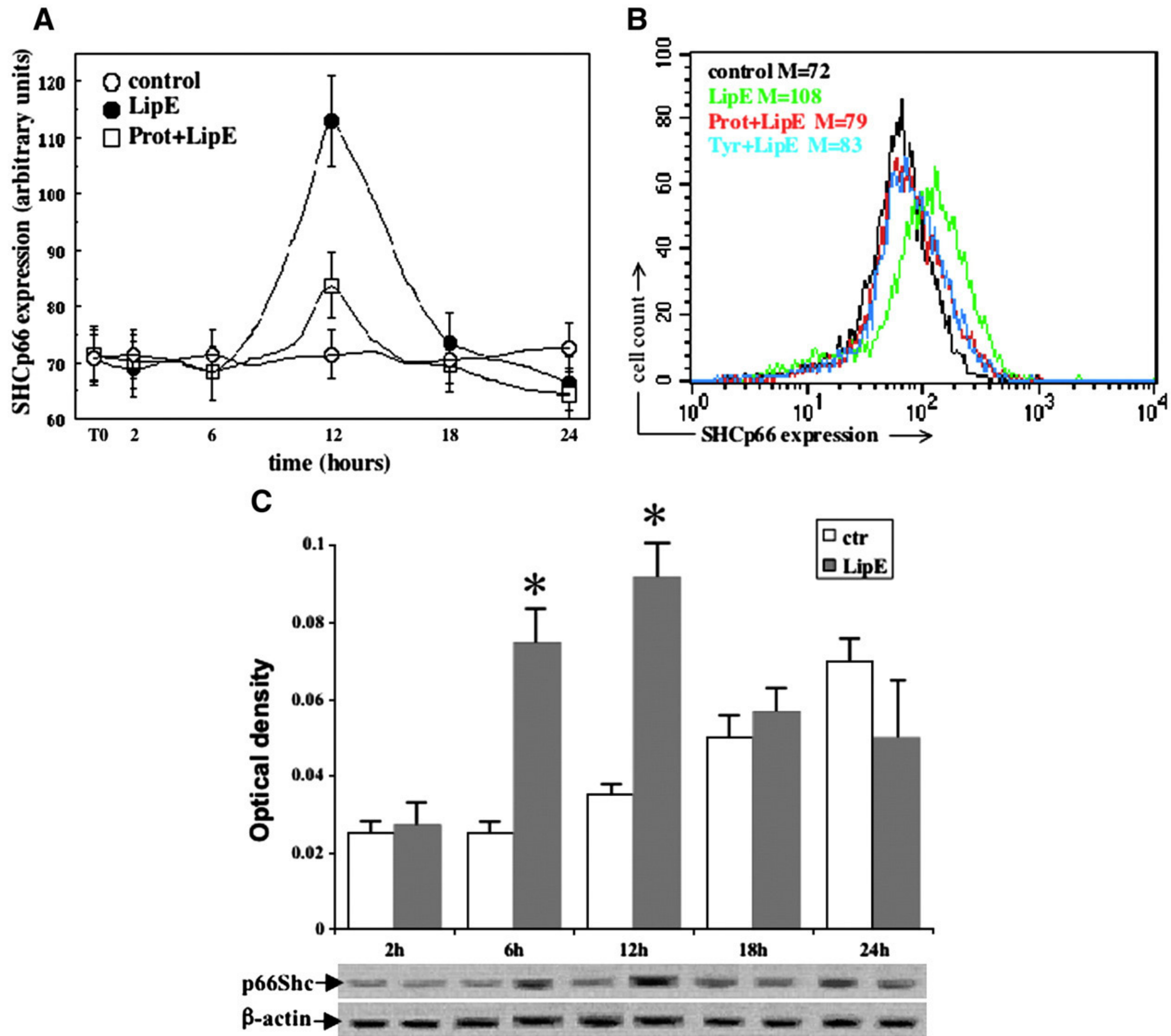

Fig. 4. p66Shc expression. (A and B) Quantitative evaluation by flow cytometry of p66Shc intracellular content. Data reported in (A) are the mean of four independent experiments±S.E.M. In ordinate axis, the median values of the fluorescence intensity histograms are reported. In (B), results obtained by a representative experiment are shown. Numbers in (B) represent median values of fluorescence intensity histograms. $P<.01$: LipE vs. control; LipE vs. PROT+LipE; LipE vs. TYR+LipE. (C) p66Shc protein evaluation performed by Western blotting and densitometric analyses throughout LipE treatment (2–24 h) of Caco-2 cells. Values are the mean±S.E.M. of four independent experiments, performed in duplicate. (*$P<.001$ vs. control, for each time point considered). The blots are representative of four independent experiments. Equal loading of proteins was verified by β-actin immunoblotting.

It has been shown that, in response to ROS overproduction, p66Shc was overexpressed and redistributed [25]. Therefore, we also investigated the intracellular localization of p66Shc (Fig. 5) under basal conditions (first row, left column) and after oxidative stress induced by oxidized lipids, in the absence (second row, left column) and in the presence (third row, left column) of antioxidant phenolic compounds at different time points. These analyses — performed by confocal microscopy after double staining of mitochondria (green fluorescence) and p66Shc (red fluorescence) — clearly indicated that (i) 12 h after exposure to oxidized lipids, p66Shc protein was redistributed and partially localized at the mitochondrial level (second row, third column, see yellow staining), whilst in untreated control cells it was localized throughout the cytoplasm (first row, third column); and (ii) treatment with antioxidants before oxidized lipid administration impaired the p66Shc rearrangement (Fig. 5, third row, results obtained with tyrosol). Importantly, p66Shc intracellular redistribution occurred 12 h after oxidized lipid administration. It is worth noting that this could represent an early apoptotic event induced by oxidized lipids. In fact, no signs of chromatin condensation or fragmentation were detectable by Hoechst staining at this time point (12 h; Fig. 5, second row, third column) in Caco-2 cells. Moreover, in the light of the literature [25], further experiments have been carried out by confocal microscopy after double labeling of p66Shc and endoplasmic reticulum (ER)-specific protein disulfide isomerase in cells treated with LipE. The results obtained are shown in Fig. 6 where a partial localization of p66Shc at ER level is visible in all the

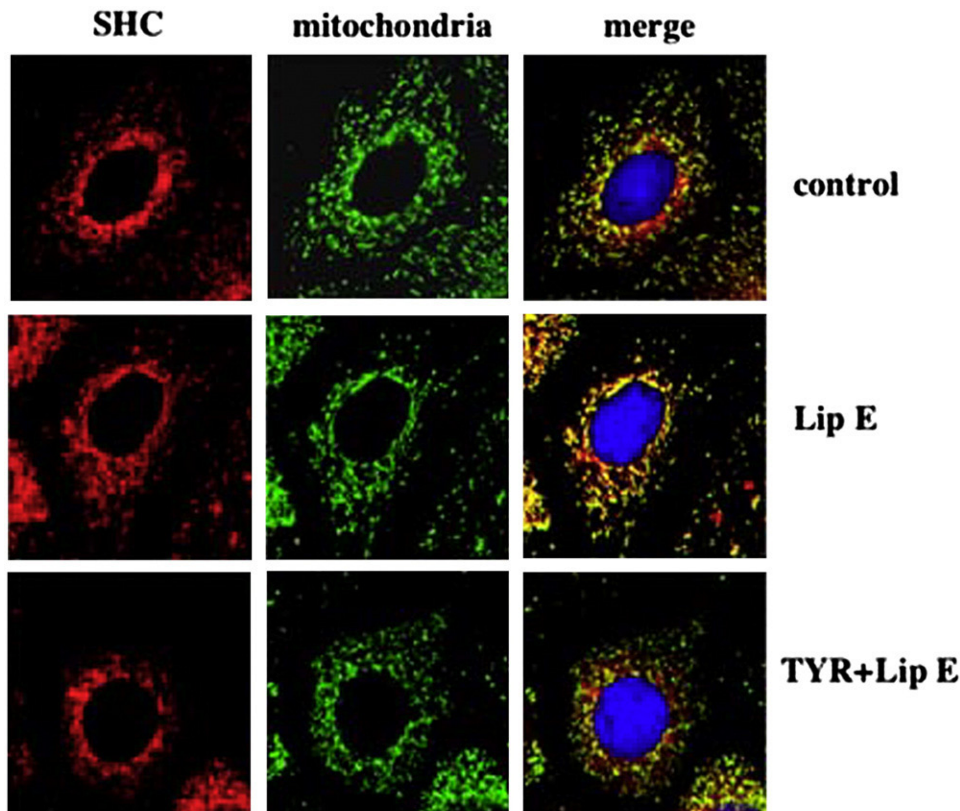

Fig. 5. Analytical cytology of p66Shc and mitochondria. Qualitative analysis by confocal microscopy technique of mitochondria (green, middle column) and p66Shc protein (red, left column). First row: control untreated cells; second row: cells exposed to LipE for 12 h; third row: cells exposed to LipE for 12 h in the presence of tyrosol (TYR+LipE). Yellow staining indicates mitochondria and p66Shc co-localization. Magnification ×2500.

experimental conditions (see yellow spots). In fact, merge pictures (right column) show a yellow staining in untreated cells (first row) as well as in LipE- (middle row) and TYR-LipE-treated cells (bottom row).

4. Discussion

The toxicity of oxLDL is mainly associated with the lipid fraction, which contains a wide variety of oxidized lipids, such as oxysterols, lipid peroxides and end products of lipid peroxidation. Oxidized lipids interacting with cell surface components, membrane lipids and proteins, or diffusing inside the cell, elicit lipid peroxidation of cellular lipids and ROS generation [26]. The altered redox balance observed in this study was characterized by intracellular ROS overproduction, extensive depletion of the GSH pool and modification of the antioxidant enzymes in terms of both *activity* and *expression*. Oxidized lipid treatment of intestinal cells resulted in a rapid and progressive overproduction of intracellular ROS as earlier event (6 h), before any decrease of intracellular glutathione (detectable after 12 h). When cells are exposed to increased oxidative stress, the GSH/GSSG ratio will decrease as a consequence of GSSG accumulation. Oxidized lipid treatment significantly reduced the GSH/GSSG ratio, clearly indicating the onset of oxidative stress. Twelve hours of exposure to oxidized lipids seemed to correspond to the critical time point beyond which cell redox balance was irreversibly impaired. SOD, CAT and GPx activities were significantly reduced while GSSGred activity, after a rapid initial decrease (6 h after exposure to oxidized lipid), increased throughout the experiment. The reduced activities of both SOD and GPx suggest a protein damage, probably involving the active sites of the two antioxidant enzymes. It has been actually demonstrated that the active site of Cu/Zn-SOD can be inactivated by H_2O_2 [27] and that ROS can oxidize an essential amino acid residue in the active center of GPx, inactivating the enzyme [27,28]. The studies on antioxidant enzyme gene expression in intestinal cells exposed to oxidized lipids showed a profound intracellular "remodeling" to occur: a rapid, long-term mRNA synthesis of the GSH-cycle enzymes GPx, GSSGred and γ-GCS was detected. Engagingly, the overexpression of GPx mRNA may indicate a reaction mechanism to counteract the increased levels of peroxides and compensate for enzyme inactivation, while up-regulation of GSSGred and γ-GCS may indicate a compensation mechanism to meet the demand of excessive glutathione in reduced form. Moreover, the activation and binding of several nuclear transcription factors to the promoter region of antioxidant GSH-related enzyme genes could also be responsible for the increased mRNA levels [29,30].

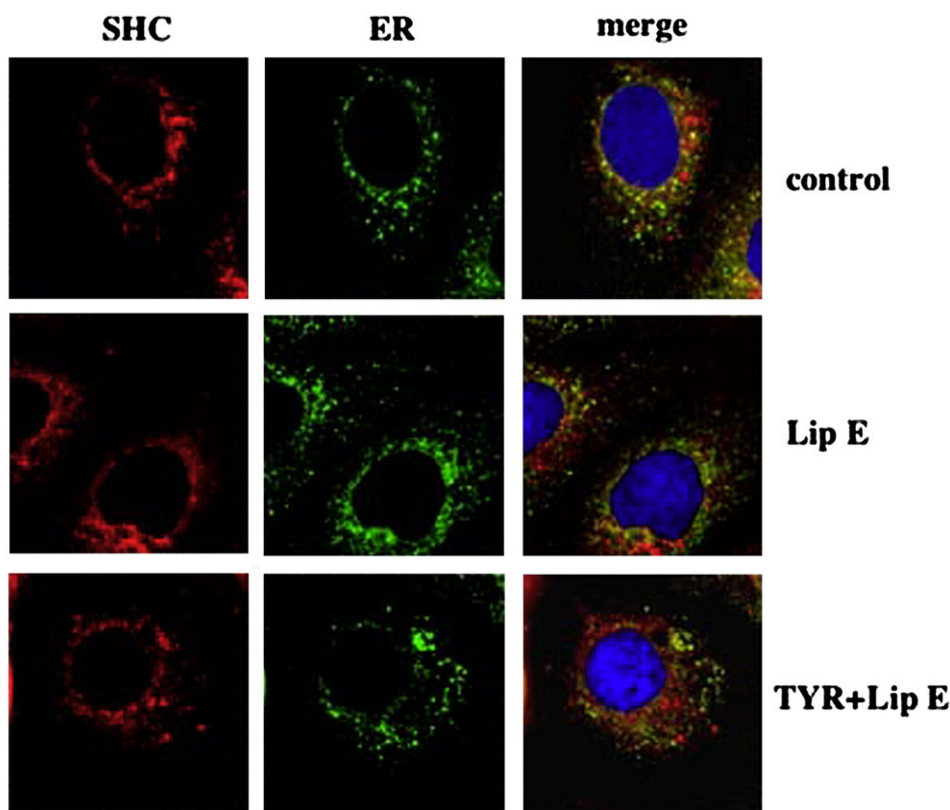

Fig. 6. Analytical cytology of p66Shc and endoplasmic reticulum. Qualitative analysis by confocal microscopy technique of ER (green, middle column) and p66Shc protein (red, left column). First row: control untreated cells; second row: cells exposed to LipE for 12 h; third row: cells exposed to LipE for 12 h in the presence of tyrosol (TYR+LipE). Note the weak co-localization in merge pictures (yellow spots). Magnification ×2500.

The involvement of ROS in apoptosis induced by different agents, such as oxidants, toxicants or drugs, was suggested by a number of studies [31,32]. For instance, the early intracellular decrease in GSH content seems to be a central event in the previously demonstrated apoptotic cell death induced by oxLDL in the enterocyte-like Caco-2 cell line [15]. P66Shc molecule has recently been suggested to play a key role in cell responses related with oxidative stress and apoptosis [33,34]. p66Shc has been identified as a splice variant of p52Shc/p46Shc, but unlike these variants (involved mainly in the submission of mitogenic signals), evidence exists that p66shc functions in the intracellular pathway converting oxidative signals into apoptosis [35]. For instance, the treatment of mouse fibroblasts with oxidants resulted in increased expression of p66Shc and apoptosis, while p66Shc−/− fibroblasts have shown that reduced apoptosis correlated with increased resistance to these oxidative stimulations [33]. The mechanisms of action of p66Shc have not been elucidated yet. It is, however, accepted that it acts as a sensor for ROS production and as the downstream target of activated p53 in p53-dependent apoptosis [35]. Interestingly, a correlation between p66Shc expression and the adverse effect of high-fat diet, such as increased LDL oxidability, was also demonstrated [36]. The present study suggests for the first time that exposure of intestinal cells to oxidized lipids increased p66Shc content when GSH levels decreased, and the antioxidant system could not buffer the overproduction of ROS any longer. Then, as suggested in other systems, i.e., primary mouse fibroblasts [25], the intrinsic program of self-destruction was activated by an increase and redistribution of p66Shc. In particular, the reported data may suggest that an increase of p66Shc mitochondrial pool could exert a role in the signaling pathway leading to oxidized lipid-induced apoptosis in human intestinal cells.

Finally, this study underlines the protective effects exerted by two dietary polyphenols in this enterocyte-like system. The protective effect exerted by phenol compounds seems to involve more than one mechanism. Not only can they scavenge free radicals, prevent lipid peroxidation by metal chelation and break peroxidative chain reactions, but they can also exert other additional effects [37]. For example, polyphenols have a direct effect on gene transcription of GSH-related enzymes, as well as on the activity of several regulatory pathways such as MAPK cascade or nuclear factor-κB as suggested by us and other authors in different cell systems [38–42]. The results of this study not only indicate that the two phenolic compounds exhibit strong antioxidant activities that protect intestinal cell from oxidative stress, but also highlight the antiapoptotic effect as a further mechanism. The sparing of GSH, i.e., the strengthening of intracellular antioxidant defences, inhibits

the intracellular signals leading to overexpression and mitochondrial recruitment of p66Shc, thus regulating intestinal cell fate resulting in cell survival.

Although the beneficial role of polyphenols in preventing degenerative diseases is well established [8,43], discrepancies between their activity in in vitro and in vivo systems have been described [44,45]. Even after extensive polyphenol intake, maximal plasma concentrations remain low without exerting any significant systemic effect. In fact, depending on the chemical structure, polyphenol absorption, metabolism and elimination are variable, and polyphenol metabolites can exert a very low antioxidant activity [46,47]. Furthermore, while the bioavailability of the polyphenols is a limiting factor in the majority of body districts [44], the ingested polyphenols, because of the enzymatic activities of enterocytes and colon bacteria [4], concentrate in their active forms in intestinal lumen. This could result in a more pronounced protective activity on the intestinal mucosa. In addition, the presence of large amounts of different phenolic compounds together with other diet antioxidants (tocopherols, carotenoids, ascorbate) in the gastroenteric tract can be highly effective in preventing the production of diet oxidative compounds, or, additionally, they can directly neutralize diet oxidants.

These results provide, for the first time, new evidence indicating the involvement of the p66Shc molecule in intestinal cell intoxication due to oxidized lipids. In addition, they further suggest that an increased polyphenol intake, through diet supplementation or consumption of food rich in these natural antioxidants, may prevent free radical-mediated intestinal cell degeneration associated with morbidity states.

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References

[1] Parks DA, Bulkley GB, Granger DN. Role of oxygen-derived free radical in digestive tract diseases. Surgery 1983;94:415–22.

[2] Grisham MB. Oxidants and free radicals in inflammatory bowel disease. Lancet 1994;344:859–61.

[3] Aw TY. Molecular and cellular responses to oxidative stress and changes in oxidation-reduction imbalance in the intestine. Am J Clin Nutr 1999;70:557–65.

[4] Dryden GW, Song M, McClain C. Polyphenols and gastrointestinal diseases. Curr Opin Gastroenterol 2006;22:166–70.

[5] Kris-Etherton PM, Keen CL. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. Curr Opin Lipidol 2002;13:41–9.

[6] Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. Biochem Biophys Res Comm 1998;247:60–4.

[7] Duthie SJ, Dobson VL. Dietary flavonoids protect human colonocyte DNA from oxidative attack in vitro. Eur J Nutr 1999;38:28–34.

[8] Manach C, Mazur A, Scalbert A. Polyphenols and prevention of cardiovascular diseases. Curr Opin Lipidol 2005;16:77–84.

[9] Moskaug JO, Carlsen H, Myhrstad MCW, Blomhoff R. Polyphenols and glutathione synthesis regulation. Am J Clin 2005;81(Suppl):277S–83S.

[10] Yu R, Jiao JJ, Duh JL, Gudehithlu K, Tan TH, Kong AN. Activation of mitogen-activated protein kinases by green tea polyphenols: potential signalling pathways in the regulation of antioxidant-responsive element-mediated phase II enzyme gene expression. Carcinogenesis 1997;18:451–6.

[11] Halliwell B, Rafter J, Jenner A. Health promotion by flavonoids tocopherols, tocotrienols and other phenols: direct or indirect effects? Antioxidant or not? Am J Clin 2005;81(Suppl):268S–76S.

[12] Chandra J, Samali A, Orrenius S. Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 2000;29:323–33.

[13] Galle J, Schneider R, Heinloth A, Wanner C, Galle PR, Conzelmann E, et al. Lp (a) and LDL induce apoptosis in human endothelial cells and in rabbit aorta: role of oxidative stress. Kidney Int 1999;55:1450–61.

[14] Giovannini C, Straface E, Modesti D, Coni E, Cantafora A, De Vincenzi M, et al. Tyrosol, the major olive oil biophenol, protects against oxidized-LDL-induced injury in Caco-2 cells. J Nutr 1999;129:1269–77.

[15] Giovannini C, Matarrese P, Scazzocchio B, Sanchez M, Masella R, Malorni W. Mitochondria hyperpolarization is an early event in oxidized low density lipoprotein-induced apoptosis in Caco-2 cells. FEBS Lett 2002;523:200–6.

[16] Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, et al. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 1983;47:323–30.

[17] Wang TG, Gotoh Y, Jennings MH, Rhoads CA, Aw TY. Lipid hydroperoxide-induced apoptosis in human colonic Caco-2 cells is associated with an early loss of cellular redox balance. FASEB J 2000;14:1567–76.

[18] Li Q, Sai Y, Kato Y, Tamai I, Tsuji A. Influence of drugs and nutrients on transporter gene expression levels in Caco-2 and LS180 intestinal epithelial cells. Pharm Res 2003;20:1119–24.

[19] Purdom S, Chen QM. P66Shc: at the crossroad of oxidative stress and the genetics of aging. Trends Mol Med 2003;9:206–10.

[20] Masella R, Giovannini C, Varì R, Di Benedetto R, Coni E, Volpe R, et al. Effects of dietary virgin olive oil phenols on low density lipoprotein oxidation in hyperlipidemic patients. Lipids 2001;36:1195–202.

[21] Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem 1957;226:497–509.

[22] Matarrese P, Tinari N, Semeraro ML, Natoli C, Iacobelli S, Malorni W. Galectin-3 overexpression protects from cell damage and death by influencing mitochondrial homeostasis. FEBS Lett 2000;473:311–5.

[23] Sahaf B, Heydary K, Herzenberg LA, Herzenberg LA. Lymphocyte surface thiols levels. Proc Natl Acad Sci U S A 2003;100:4001–5.

[24] Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469–74.

[25] Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA, Piccini D, et al. The life span determinant p66Shc localizes to mitochondria where it associates with mtHsp70 and regulates trans-membrane potential. J Biol Chem 2004;279:25689–95.

[26] Salvayre R, Auge N, Benoist H, Negre-Salvayre A. Oxidized low-density lipoprotein-induced apoptosis. Biochim Biophys Acta 2002;1585:213–21.

[27] Mates JM. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 2000;153:83–104.

[28] Fujii J, Taniguchi N. Down regulation of superoxide dismutases and glutathione peroxidase by reactive oxygen and nitrogen species. Free Radic Res 1999;31:301–8.

[29] Bea F, Hudson FN, Chait A, Kavanagh TJ, Rosenfeld ME. Induction of glutathione synthesis in macrophages by oxidized low-density lipoproteins is mediated by consensus antioxidant response elements. Circ Res 2003;92:386–93.

[30] Zhou LZ, Johnson AP, Rando TA. NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. Free Radic Biol Med 2001;31:1405–16.

[31] Chandra J, Samali A, Orrenius S. Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 2000;29:323–33.

[32] Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, Yodoi J. Redox control of cell death. Antioxid Redox Signal 2002;4:405–14.

[33] Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, et al. The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 1999;402:243–5.

[34] Pacini S, Pellegrini M, Migliaccio E, Patrussi L, Ulivieri C, Ventura A, et al. p66SHC promotes apoptosis and antagonizes mitogenic signaling in T cells. Mol Cell Biol 2004;24:1747–57.

[35] Trinei M, Giorgio M, Cicalese A, Barozzi S, Ventura A, Migliaccio E, et al. A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. Oncogene 2002;21:3872–8.

[36] Napoli C, Martin-Padura I, de Nigris F, Giorgio M, Mansueto G, Somma P, et al. Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet. Proc Natl Acad Sci U S A 2003;100:2112–6.

[37] Masella R, Di Benedetto R, Varì R, Filesi C, Giovannini C. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. J Nutr Biochem 2005;16:577–86.

[38] Masella R, Varì R, D'Archivio M, Di Benedetto R, Matarrese P, Malorni W, et al. Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. J Nutr 2004;134:785–91.

[39] Carlsen H, Myhrstad MC, Thorensen M, Moskaug JO, Blomhoff R. Berry intake increases the activity of the γ-glutamylcysteine synthetase promoter in transgenic reporter mice. J Nutr 2003;133: 2137–40.

[40] Myhrstad MCW, Carlsen H, Nordstrom O, Blomhoff R, Moskaug JO. Flavonoids increase the intracellular glutathione level by trans-activation of the γ-glutamylcysteine synthetase catalytic subunit promoter. Free Radic Biol Med 2002;32:386–93.

[41] Chen C, Yu R, Owuor ED, Kong AN. Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenols components during cell survival and death. Arch Pharm Res 2000;23:605–12.

[42] Kong AN, Owuor E, Yu R, Hebbar V, Chen C, Hu R, et al. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metab Rev 2001;33:255–71.

[43] Scalbert A, Manach C, Morand C, Remesy C, Jimenez L. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr 2005;45:287–306.

[44] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans: I. Review of 97 bioavailability studies. Am J Clin 2005;81(Suppl):230S–42S.

[45] Williamson G, Manach C. Bioavailability and bioefficacy of polyphenols in humans: II. Review of 93 bioavailability studies. Am J Clin 2005;81(Suppl):230S–42S.

[46] Depeint F, Gee JM, Williamson G, Johnson IT. Evidence for consistent patterns between flavonoid structures and cellular activities. Proc Nutr Soc 2002;61:97–103.

[47] Clifford MN. Diet-derived phenols in plasma and tissues and their implications for health. Planta Med 2004;70(12):1103–14.