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Mitochondria hyperpolarization is an early event in oxidized low-density lipoprotein-induced apoptosis in Caco-2 intestinal cells

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Abstract **We investigated the mechanisms underlying the pro-apoptotic activity exerted by oxidized low-density lipoproteins (oxLDL) in Caco-2 intestinal cells, a cell line which retains many morphological and enzymatic features typical of normal human enterocytes. We found that: (i) oxLDL induced mitochondrial-mediated apoptosis by provoking first an increase in mitochondrial membrane potential, followed, later, by the typical apoptosis-associated depolarization (type II apoptosis); accordingly, (ii) caspase-9 inhibition significantly hindered apoptosis while caspase-8 inhibition did not; and finally (iii) dietary phenolic antioxidizing compounds exerted a significant protective antiapoptotic activity. These results point to mitochondrial hyperpolarization as 'sensitizing feature' in apoptotic proneness of Caco-2 intestinal cells to oxLDL exposure.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Caco-2; Mitochondrial membrane potential; Apoptotic pathway; oxLDL

1. Introduction

The complex cascade of events involving pro-apoptotic or antiapoptotic signals depend upon, or are associated with, the type of the 'trigger' that activates the process [1]. A hypothesis regarding two different cell types characterized by different apoptotic pathways has in fact been proposed. These two cell pathways are referred to different initiation patterns [2]. In particular, caspase-9-driven apoptotic machinery seems to mainly involve mitochondrial-associated caspase cascade, while caspase-8-mediated apoptosis appears to be of importance in the receptor-mediated, i.e. Fas-mediated, cell death [2].

Dysregulated apoptosis is actually found in a number of pathologic conditions of the gastrointestinal tract [3,4]. Being the interface between the organism and its luminal environment, the intestine is in fact constantly challenged by diet-derived oxidants as well as by endogenously generated reactive oxygen species (ROS) or oxidants. In particular, a high intake of dietary polyunsaturated fatty acids can contribute to the luminal accumulation of lipid hydroperoxides and, subsequently, lipid peroxidation products that can induce oxidative stress and redox imbalance [5,6]. In the same vein, low-density lipoproteins undergoing oxidative modification (oxLDL), generate a mixture of compounds with cytotoxic activity, such as products of lipid peroxidation (4-hydroxy-2,3-nonenal, lipid hydroperoxides and oxysterols). In turn, the interaction of oxLDL-derived oxidizing products with cells and tissues results in oxidative imbalance leading to cell death by apoptosis [7,8]. Oxidative stress has been associated with many forms of programmed cell death and there is strong evidence that ROS can induce the apoptotic process. On the other hand, it is generally accepted that antioxidizing drugs can prevent apoptosis [9]. Increased attention has been recently devoted to phenolic compounds, antioxidants that occur naturally in vegetables, fruits and beverages [10,11] such as tea, red wine and extra virgin olive oil, because of their possible beneficial role in the prevention of several oxidative stress-associated diseases, which are characterized by inflammatory injuries, including injury of intestine [12].

Aim of the present work was to investigate the mechanisms underlying oxLDL-induced apoptosis in Caco-2 cells which, retaining many of the morphological and enzymatic features typical of normal human enterocytes [13], are largely used as a model system for evaluating the effects of normal dietary constituents as well as additives, contaminants, toxicants, oxidants and drugs [12,14,15].

2. Materials and methods

2.1. LDL isolation and oxidation

LDL (1.019–1.063 g/ml) were prepared from freshly isolated pooled plasma from different healthy human donors by density gradient ultracentrifugation and than oxidized with 25 μM $CuSO_4$ for 18 h at 37°C as previously reported [10]. The extent of lipid peroxidation was estimated as malondialdehyde and 4-hydroxyalkenals content by a colorimetric kit (LPO 586, Bioxytech S.A., Bonneuil sur Marne, France). The results showed a mean value of 50.0 ± 8.2 nmol/mg

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Abbreviations: DEVD-CHO, Asp-Glu-Val-Asp-aldehyde; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; DHR 123, dihydrorhodamine 123; $\Delta\Psi$, mitochondrial membrane potential; FCCP, carbonyl-cyanide *p*-(trifluoro-methoxy) phenylhydrazone; HE, hydroetidine; IETD-CHO, Ile-Glu-Thr-Asp-aldehyde; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolcarbocyanineiodide; LDL, low-density lipoproteins; LEHD-CHO, Leu-Glu-His-Asp-aldehyde; oxLDL, oxidized low-density lipoproteins; ROS, reactive oxygen species; TMRM, tetramethylrhodamine ester

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LDL protein in oxidized samples versus 4 ± 0.05 nmol/mg protein in native LDL. Modification of LDL was tested by measuring the increase in electrophoretic mobility on 0.5% agarose gel, and the fragmentation of apo B by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.2. Cell cultures

The human colon cell line Caco-2 was obtained from the European Collection of Cell Culture (Salisbury, UK). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Cramlington, UK) with 4.5 g glucose per l, supplemented with 1% (v/v) non-essential amino acids (Flow Laboratories, Irvine, UK), 0.2 mM L-glutamine (Flow), 50 U/ml penicillin (Flow), 50 μg/ml streptomycin (Flow), and 10% (v/v) fetal calf serum (Flow), at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.3. Cell treatments

LDL: native LDL or oxLDL were sterilized by filtration through a 0.2 μm Millipore membrane (Millipore Corporation, Bedford, MA, USA) and added after 5 days culture at a final concentration of 0.2 mg/ml as previously reported [10] for 6, 12, 18, 24 and 48 h. The same LDL preparations at the same concentration, without exposure to copper, were used as native LDL. *Fas*: cell cultures were incubated with (i) 15 ng/ml anti-Fas activating antibody (clone CH11); (ii) 1 μg/ml anti-Fas inhibiting antibody (clone ZB4) (Upstate Biotechnology, Lake Placid, NY, USA) 2 h before exposure to oxLDL; (iii) 2 μg/ml anti-FasL inhibiting antibody (clone NOK-1) (Pharmingen, San Diego, CA, USA) 2 h before exposure to oxLDL. As further control, mouse IgM (control for Fas triggering), mouse IgG_1 (control for Fas and for FasL neutralizing) were also considered. *Phenols*: tyrosol (0.5 mM) and protocatechuic acid (0.25 mM) were administered in the sterile medium 2 h before exposure to oxLDL and maintained in the culture medium during treatments. *Caspase inhibitors*: 2 h before the apoptotic stimuli described above, 100 μM Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) (cell permeant caspase-3 inhibitor), LEHD-CHO (cell permeant caspase-9 inhibitor), or Ile-Glu-Thr-Asp-aldehyde (IETD-CHO) (cell permeant caspase-8 inhibitor) (Biosource International, Camarillo, CA, USA) were directly added to the culture medium. Cells treated with caspase inhibitors alone were considered as controls. Carbonyl-cyanide *p*-(trifluoro-methoxy) phenylhydrazone (FCCP): at the same time of oxLDL administration, 40 nM of FCCP, an uncoupler of mitochondrial oxidation and phosphorylation, was added to the culture medium. Cells treated with oxLDL alone or FCCP alone were considered as control of this series of experiments.

2.4. Proliferating activity

Cells were exposed to LDL or oxLDL 5 days after seeding in the presence or absence of tyrosol or protocatechuic acid. After the incubation period, 1.85 kBq of [^{14}C]thymidine (Amersham, Buckinghamshire, UK; special activity: 2.09 GBq/mmol) were added to each well. After a 4-h [^{14}C]thymidine incubation, cells were prepared as previously reported [10]. Radioactivity was evaluated in 0.2-ml aliquots of NaOH extracts with a liquid scintillation spectrometer. [^{14}C]thymidine incorporation was expressed as a percentage of values observed in untreated cells.

2.5. Apoptosis evaluation

Apoptosis was evaluated with the ApoAlert Annexin V apoptosis kit (Clontech Laboratories, Palo Alto, CA, USA) following the manufacturer's instructions. Two-color cytometric analysis (fluorescence-activated cell sorting (FACS)) was performed on a Coulter Epics Elite ESP cell Sorter (Miami, FL, USA) with an argon-ion laser tuned at 488 nm.

2.6. Measurement of caspase-3 activity

Activity of caspase-3 was measured by a commercial kit ApoAlertTM (Clontech Laboratories, Palo Alto, CA, USA). The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*-NA) after cleavage from the labeled substrates. The *p*-NA light emission can be quantified using a microtiter plate reader at 405 nm (Novopath, Bio-Rad, Hercules, CA, USA). The fold increase in caspase activity was determined by comparing the absorbance of *p*-NA from apoptosis-induced samples vs. uninduced control samples [16].

2.7. Intracellular redox state

Control and treated cells (5×10^5) were harvested and incubated in 495 μl of Hanks' balanced salt solution (HBSS, pH 7.4) with 5 μl of hydroetidine (HE, Molecular Probes) or dihydrorhodamine 123 (DHR 123, Molecular Probes) in polypropylene test tubes for 15 min at 37°C. The final concentration of HE and DHR 123 were 1 μM and 10 μM, respectively. HE is a non-fluorescent membrane-permeable ethidium derivative that can be oxidized directly to the red fluorescent ethidium bromide (EB) by O_2^- generated inside the cells

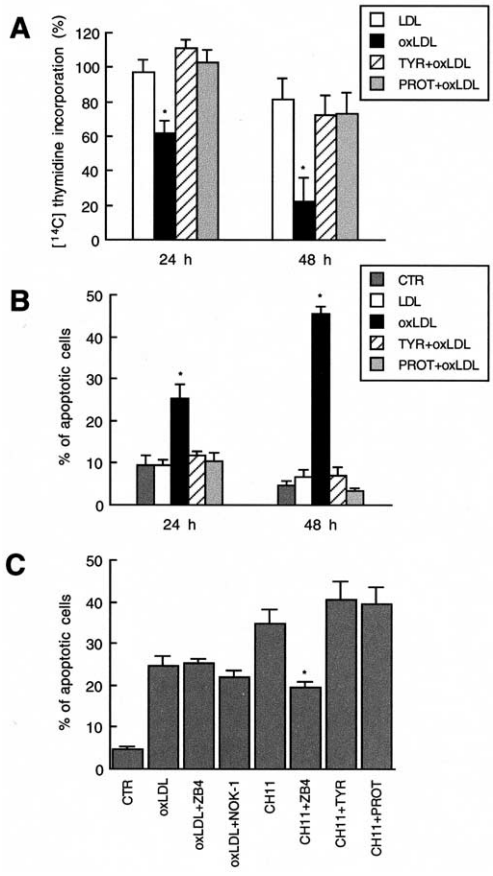

Fig. 1. Cytostatic activity and pro-apoptotic effects of oxLDL in intestinal cells. A: Relative incorporation of [^{14}C]thymidine by Caco-2 cells exposed to native LDL or oxLDL (0.2 mg/ml) in the presence or absence of phenolic compounds, 0.5 mM tyrosol (TYR) or 0.25 mM protocatechuic acid (PROT). Each column represents the relative incorporation after 24 and 48 h treatment as compared with control values (100%). The data are expressed as mean ± S.E.M. of four independent experiments. *$P < 0.001$: oxLDL vs. LDL; oxLDL vs. TYR+oxLDL; oxLDL vs. PROT+oxLDL. B: Percentage of apoptotic annexin-V positive cells in control cells (CTR), native LDL exposed cells (LDL), oxLDL-treated cells (oxLDL) and oxLDL-treated cells preincubated with tyrosol (TYR+oxLDL) or protocatechuic acid (PROT+oxLDL). Values are expressed as mean ± S.E.M. of six independent experiments. *$P < 0.001$: oxLDL vs. LDL; oxLDL vs. TYR+oxLDL; oxLDL vs. PROT+oxLDL. C: Quantification of apoptosis in Caco-2 cells incubated with (i) anti-Fas activating antibody (clone CH11); (ii) anti-Fas activating antibody (clone CH11)+anti-Fas inhibiting antibody (clone ZB4); (iii) anti-FasL inhibiting antibody (clone NOK-1) 2 h before oxLDL exposure; (iv) anti-Fas inhibiting antibody (clone ZB4) 2 h before oxLDL exposure; (v) anti-Fas activating antibody (clone CH11)+tyrosol; (vi) anti-Fas activating antibody (clone CH11)+protocatechuic acid. The percentages of annexin-V positive cells 24 h after apoptotic triggering are reported as mean ± S.E.M. of four independent experiments. *$P < 0.001$: anti-Fas activating antibody (clone CH11)+anti-Fas inhibiting antibody (clone ZB4) vs. anti-Fas activating antibody (clone CH11).

after different treatments. DHR 123 is a dye freely diffusing into cells which is oxidized primarily by H_2O_2 in a myeloperoxidase-dependent reaction to green fluorescence. As these probes can be accumulated by mitochondria as a function of their membrane potential, we excluded a possible interference of the different mitochondrial membrane potential state showed by variously treated cells with ROS evaluation by using FCCP (20 μM, Sigma) a protonophore that, at this dose, completely depolarized the mitochondrial membrane, as previously described [17]. Intracellular GSH content was assessed by using 10 μM 5-chloromethyl-2′,7′-dichloro-dihydrofluoresceindiacetate (CM-H_2DCFDA, Molecular Probes). Cells exposed to L-buthionine-[S,R]-sulfoximine 7.5 mM (BSO, Sigma), a GSH depleting drug, for 16 h were considered as negative controls [17].

2.8. Mitochondrial membrane potential (ΔΨ) and mitochondrial mass

The $\Delta\Psi$ of control and treated cells was studied by using: (i) the 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolcarbocyanineiodide (JC-1) probe (5 μM, Molecular Probes) [18], (ii) the 3,3′-dihexyloxacarbocyanine iodide (DiOC6, 500 nM, Molecular Probes), and (iii) the tetramethylrhodamine ester (TMRM, 1 μM, Molecular Probes). In consideration of the not significant differences obtained with these probes, only results obtained with JC-1 will be shown. As negative and positive methodological controls, cells were also incubated for 15 min with 5 mM oligomycin (which induces hyperpolarization of mitochondria) or with 10 mM valinomycin (which induces depolarization of mitochondria), respectively. For the analysis of the mitochondrial mass, cells were incubated at 37°C for 30 min with 5 μM nonylacridine orange (NAO, Molecular Probes). Samples were immediately analyzed by a Becton-Dickinson flow cytometer.

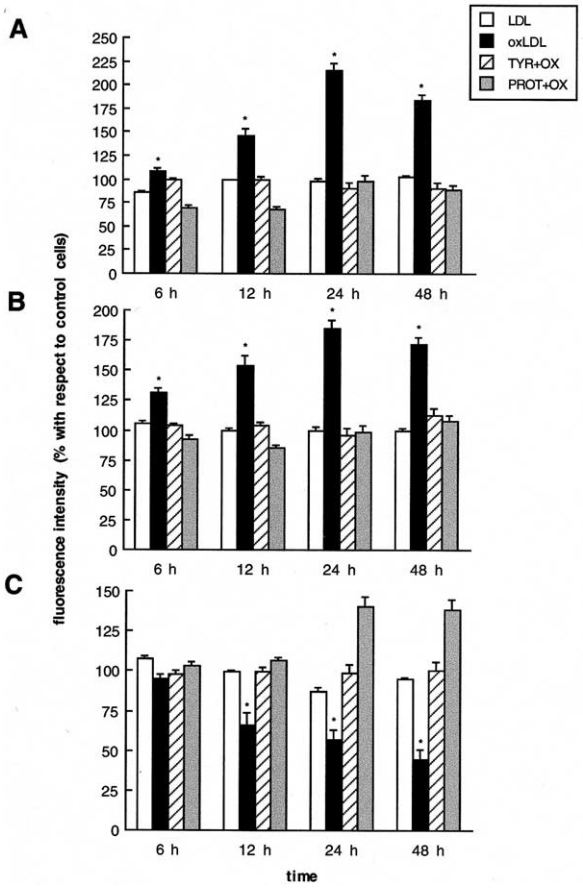

Fig. 2. OxLDL induce redox imbalance in intestinal cells. Cytofluorimetric analysis of (A) superoxide anion production, (B) hydrogen peroxide production and (C) intracellular reduced glutathione after 6, 12, 24 and 48 h exposure to LDL and oxLDL, with or without the presence of tyrosol (TYR+oxLDL) or protocatechuic acid (PROT+oxLDL). Values reported represent the mean (from four independent experiments) ± S.E.M. 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 (considered as 100%). *$P < 0.001$: oxLDL vs. LDL; oxLDL vs. TYR+oxLDL; oxLDL vs. PROT+oxLDL for each parameter and time considered.

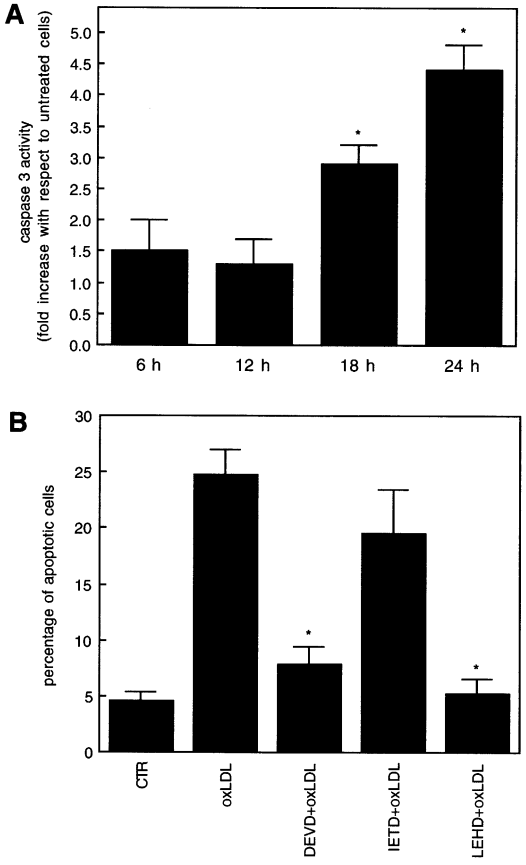

Fig. 3. OxLDL-induced apoptosis is mediated by caspase- 9 and -3. A: Activity of caspase-3 in oxLDL-treated cells. Reported values were obtained by considering the difference between caspase-3 activity found in oxLDL-treated cells and in untreated control cells. *$P < 0.001$: 18 h and 24 h oxLDL treatment vs. untreated control cells. B: Quantification of oxLDL-induced apoptosis in the absence and in the presence of DEVD-CHO (caspase-3 inhibitor), LEHD-CHO (caspase-9 inhibitor), or IETD-CHO (caspase-8 inhibitor). Values are expressed as mean ± S.E.M. of four independent experiments. *$P < 0.01$: oxLDL vs. DEVD-CHO+oxLDL; oxLDL vs. LEHD-CHO+oxLDL.

2.9. Statistical analysis

All data reported are the arithmetic mean from four independent experiments performed in triplicate ± S.E.M. Statistical calculations were performed using a one-way ANOVA. Differences among groups were examined using Bonferroni's *t*-test. Only *P* values < 0.01 were considered as significant.

3. Results

3.1. Cytostatic activity and pro-apoptotic effects of oxLDL in intestinal cells

We first evaluated subcellular activity of oxLDL by studying their effects on cell proliferation. We found (Fig. 1A) that native LDL did not induce significant changes in intestinal cell proliferation while oxLDL (0.2 mg/ml) induced a significant cytostatic effect, which was already evident after 24 h (60.6 ± 7%), and quite strong after 48 h (22.9 ± 8%). This

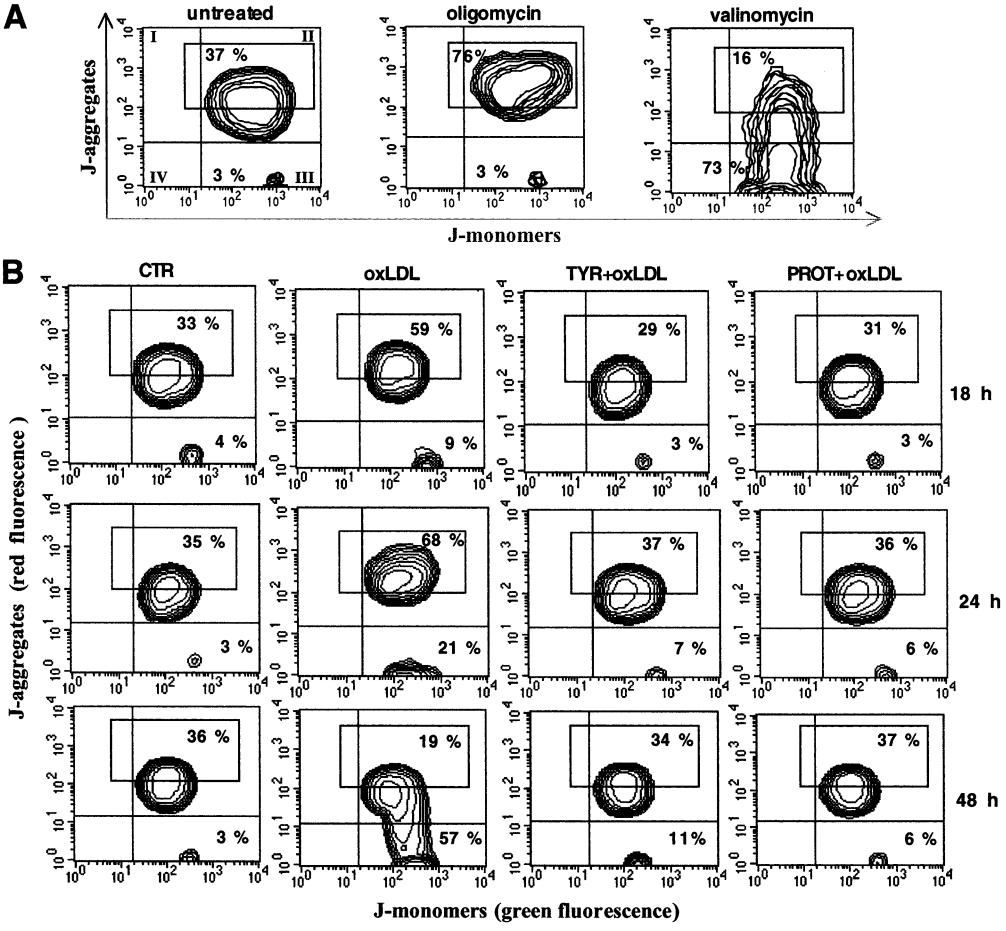

Fig. 4. OxLDL induce mitochondrial membrane hyperpolarization. Mitochondrial membrane potential assay obtained by cytofluorimetric analysis with JC-1 probe. A: Methodological controls: untreated cells (contour plot on the left), cells treated with oligomycin (hyperpolarized mitochondria, contour plot in the middle) and cells treated with valinomycin (depolarized mitochondria, contour plot on the right). B: First column, untreated control Caco-2 cells (CTR); second column, oxLDL-treated cells (oxLDL); third column, cells treated with tyrosol and oxLDL (TYR+oxLDL); fourth column, cells treated with protocatechuic acid and oxLDL (PROT+oxLDL). Values in the boxes indicate the percentage of cells that express high red fluorescence (corresponding to J-aggregates), which typically increases when the mitochondrial membrane becomes hyperpolarized. Percentages in quadrant III are referred to the number of cells with depolarized mitochondria. A representative experiment of four is shown.

highly significant decrease in thymidine uptake ($P < 0.001$) was fully counteracted by the two dietary antioxidizing active drugs tyrosol and protocatechuic acid (Fig. 1A). A time-course analysis was also performed to evaluate apoptosis. As reported in Fig. 1B, a significant increase in the percentage of apoptotic cells was found after oxLDL administration with respect to control cells ($26 \pm 4\%$ and $40 \pm 6\%$ after oxLDL exposure for 24 and 48 h, respectively). As for the proliferation results reported above, no pro-apoptotic activity was exerted by native LDL. OxLDL effects were significantly counteracted by cell treatment with tyrosol and protocatechuic acid (Fig. 1B). To evaluate the contribution of the 'classical' receptor-mediated cell death pathway in oxLDL-induced apoptosis, previously reported for other redox stimuli [19], Caco-2 cells were incubated with anti-Fas activating antibody. As controls, anti-Fas neutralizing antibodies or anti-FasL neutralizing antibodies in the presence or the absence of oxLDL were analyzed. The data obtained are reported in Fig. 1C. Unlike other epithelial and intestinal cell lines, anti-Fas activating antibody triggered apoptosis of Caco-2 cells. When intestinal cells were exposed to oxLDL, the presence of anti-Fas neutralizing antibody as well as anti-FasL neutralizing antibody, did not counteract the pro-apoptotic effects of oxLDL. More importantly, the phenolic compounds did not exert any protective effect against Fas-triggered apoptosis. These results clearly indicated that (i) Fas/FasL activation was not involved in oxLDL-induced apoptosis and that (ii) phenolic drugs exerted their antiapoptotic activity against mitochondrial-mediated (caspase-9-dependent) apoptosis only, while were ineffective against Fas-mediated cascade (caspase-8-dependent).

3.2. OxLDL induce redox imbalance in intestinal cells

Considering that cell death by apoptosis can represent the final event of a redox imbalance, time-course experiments evaluating the intracellular production of ROS (Fig. 2A and B), and the GSH content (Fig. 2C) were carried out. Fig. 2A reports the production of O_2^- after 6, 18, 24 and 48 h LDL or oxLDL treatments, in the presence or absence of tyrosol and protocatechuic acid. The production of intracellular O_2^- increased early after exposure to oxLDL (6 h) reaching a plateau within 48 h. Treatment with antioxidants fully counteracted superoxide anion production, and restored O_2^- control values (Fig. 2A). A similar behavior was found upon measur-

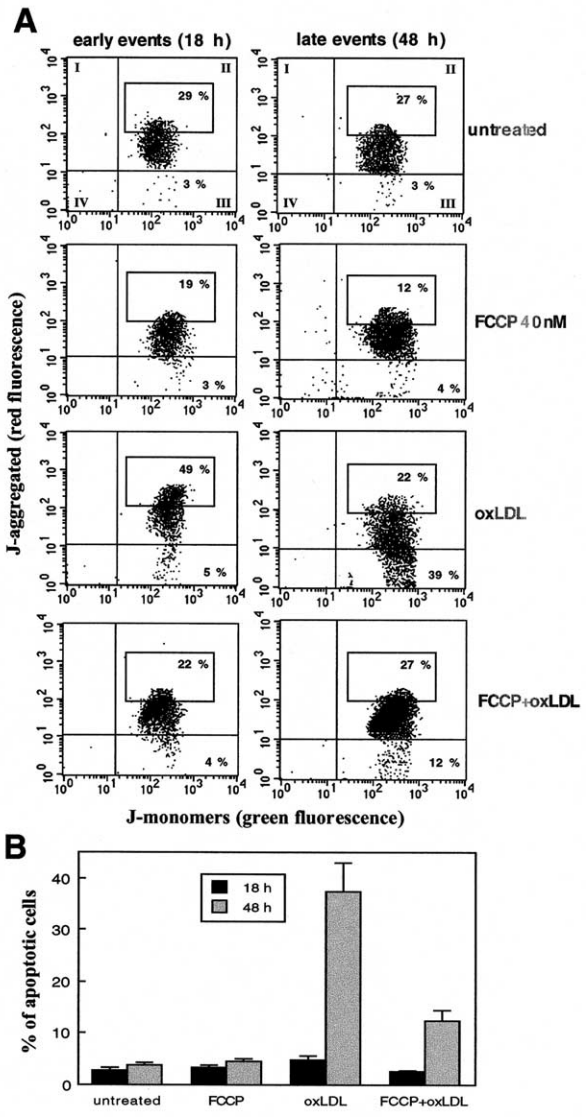

Fig. 5. Mitochondrial membrane hyperpolarization is a prerequisite for oxLDL-induced apoptosis. A: Mitochondrial membrane potential assay obtained by cytofluorimetric analysis with JC-1 probe. CTR, untreated control Caco-2 cells; oxLDL, oxLDL-treated cells; FCCP, cells treated with FCCP; FCCP+oxLDL, cells treated with FCCP and oxLDL at the same time. Values in the boxes indicate the percentage of cells that express high red fluorescence (corresponding to J-aggregates), which typically increases when the mitochondrial membrane becomes hyperpolarized. Percentages in quadrant III are referred to the cells with depolarized mitochondria. A representative experiment of four is shown. B: Percentage of apoptotic annexin-V positive cells in the same samples. Values are expressed as mean ± S.E.M. of three independent experiments. Note that a significant difference $*P < 0.001$ was detectable after 48 h of oxLDL treatment vs. FCCP given alone or vs. FCCP/oxLDL treatment.

ing hydrogen peroxide production (Fig. 2B). In fact, under the same experimental conditions, an early significant increase in H_2O_2 production was found 6 h after oxLDL administration. This effect was fully counteracted by the administration of phenolic drugs. By contrast, GSH content was significantly decreased only 12 h after treatment. The depletion was of about 50% within 48 h. The presence of the phenolic compounds restored GSH normal values. Notably, powerful beneficial effects were mainly exerted by the treatment with protocatechuic acid, probably due to its chemical structure [20].

3.3. OxLDL-induced apoptosis is mediated by caspase-9 and -3

Two different apoptotic caspase cascades, i.e. membrane-associated or mitochondria-associated, have been described. These involve caspase-8 and -9, respectively [2,21]. Both pathways converge to activate downstream events such as caspase-3 cleavage. Time-course experiments using a spectroflourimetric method indicated that intestinal cells treated with oxLDL displayed a caspase-3 activity which was two- and four-fold higher than in control samples after 18 and 24 h respectively (Fig. 3A). Moreover, the presence of the specific caspase-3 inhibitor DEVD-CHO (100 μM) reduced apoptosis by about 85% with respect to cells treated with oxLDL alone (Fig. 3B). The analysis of the effects of caspase-8 and caspase-9 inhibitors on oxLDL-induced apoptosis was performed. The results obtained indicated that LEHD-CHO (caspase-9 inhibitor), but not IETD-CHO (caspase-8 inhibitor), significantly ($P > 0.01$) inhibited oxLDL-induced apoptosis (Fig. 3B).

3.4. OxLDL induce mitochondrial membrane hyperpolarization

On the basis of the above results we hypothesize that oxLDL induced apoptosis in Caco-2 intestinal cells via a direct effect on mitochondria. To verify this hypothesis we analyzed the mitochondrial membrane potential ($\Delta\Psi$) during specific time-course experiments by using JC-1. As showed in Fig. 4A, this probe was able to reveal either an increase (induced by oligomycin, middle panel) or a decrease (induced by valinomycin, right panel) of mitochondrial membrane potential. Results reported in Fig. 4B clearly show that an early peculiar change occurred in oxLDL-treated cells. In fact, a significant percentage of oxLDL-treated cells (59 ± 5%) showed, as early event, a mitochondrial membrane hyperpolarization (Fig. 4B, upper row). This change was followed, as late event, by the characteristic drop of mitochondrial membrane potential typical of apoptosis (Fig. 4B, middle panels 24 h, bottom panels 48 h). Interestingly, phenolic compounds were able to inhibit either early (hyperpolarization) or late (depolarization) oxLDL-induced mitochondrial effects, maintaining the mitochondrial $\Delta\Psi$ values similar to those of control cells. Overlapping results were also obtained by using alternative probes DiOC6 or TMRM.

3.5. Mitochondrial membrane hyperpolarization is a prerequisite for oxLDL-induced apoptosis

To explore the possible causal link between mitochondria hyperpolarization phenomenon and apoptosis in our experimental system, a non-cytotoxic dose ('baby dose', 40 nM) of FCCP, was added to the culture medium at the same time of oxLDL. This concentration of FCCP did not induce any cytotoxic effect (the viability of cells remained > 90%, see Fig. 5B). Interestingly, FCCP was able to prevent either the initial increase (18 h, left panels of Fig. 5A) or the subsequent decline (48 h, right panels of Fig. 5A) of $\Delta\Psi$ induced by oxLDL. More importantly, FCCP also suppressed oxLDL-induced apoptosis (Fig. 5B) indicating that the early hyperpolarization of mitochondrial membrane represents a necessary event for oxLDL-induced apoptosis. Finally, the same model system was also employed to better characterize the target effect of phenolic compounds on mitochondria. In particular, we conducted specific experiments by treating cells with FCCP in the

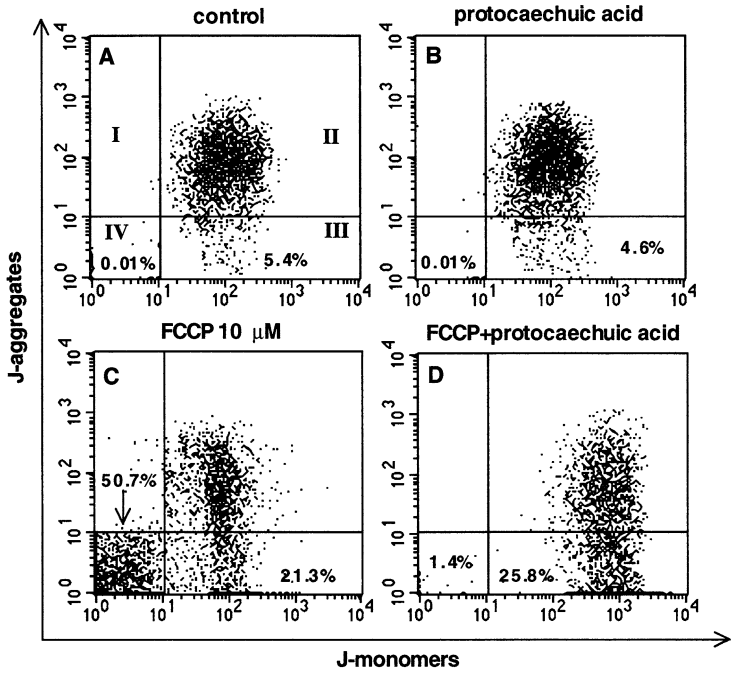

Fig. 6. FCCP-mediated mitochondrial membrane depolarization is inhibited by protocatechuic acid. Mitochondrial membrane potential assay obtained by cytofluorimetric analysis with JC-1 probe. Protocatechuic acid did not induce per se any mitochondrial depolarization in Caco-2 cells (B). By contrast, high concentrations of FCCP (10 μM) induced a high percentage of cells with partially (quadrant III) or completely (quadrant IV) depolarized mitochondria (C). Protocatechuic acid was capable of significantly reducing the number of FCCP-treated cells with depolarized mitochondria (D). A representative experiment of three is shown here.

presence or absence of phenolic compounds (Fig. 6). We found that: (i) given alone, they did not induce per se mitochondrial depolarization typical of the uncouplers (Fig. 6B), (ii) no additive effects FCCP (baby dose, 40 nM)/phenols were observed (not shown) and, more importantly, (iii) in the presence of these agents, the uncoupler activity exerted by FCCP at a higher concentration (10 μM) was significantly hindered (compare Fig. 6C and D, quadrants III and IV). In particular, the number of cells with depolarized mitochondria after FCCP administration dropped from 72.0 ± 3 to 27.1 ± 5% in the presence of protocatechuic acid and down to 28.3 ± 4% in the presence of tyrosol. In consideration of the fact that the results were quite similar, we only show the analytical cytology results obtained with protocatechuic acid (Fig. 6).

4. Discussion

Apoptosis was hypothesized to be mediated by two pathways that, although strictly intertwined, may represent different ways to reach the same result, i.e. the death of a cell [2,22]. The first pathway involves the cells that, via a death receptor pathway, e.g. by Fas, and upon caspase-8 activation, undergo apoptotic cascade. This pathway, via bid cleavage, only involves mitochondria later and finally leads to the execution phase with the cleavage of caspase-3 and poly-ADP-ribose-polymerase. A second way of caspase activation was shown to be primarily linked to mitochondrial changes, directly inducing the release of cytochrome *c* into the cytosol, and apoptosome complex formation with activation of caspase-9 [1,23,24]. Importantly, in both apoptotic pathways mitochondrial membrane depolarization has been observed [2]. By contrast, the earlier event observed in intestinal cells after oxLDL exposure was the hyperpolarization of mitochondria. This event, described by other authors for other cell types, i.e. lymphocytes, seems to represent a prerequisite for rapid mitochondrial-mediated apoptotic cell death that eventually leads to the loss of mitochondrial membrane potential [25,26]. This could, at least partially, be related to the oxidative imbalance we found after oxLDL administration in intestinal cells. The involvement of ROS species in apoptosis induced by different agents, such as oxidants, toxicants or drugs, was in fact suggested by a number of studies. Moreover it has been demonstrated that GSH depletion favors oxidative stress with the induction of apoptosis [27,28]. It can be supposed that GSH depletion is due to active GSH extrusion from apoptotic cells, or that the cellular oxidized form of glutathione (GSSG) increased as a consequence of ROS production occurring during apoptosis. Consequently, thiol-supplying drugs, e.g. *N*-acetyl-cysteine, were proved capable of protecting certain cell types from apoptosis [29]. In the same vein, our experimental findings seem to suggest that early ROS production, GSH depletion and membrane hyperpolarization, can be related events or, conceivably, according to literature data, the last could be the consequence of the first two [30]. Interestingly, phenolic drugs protect from oxLDL apoptosis and inhibit mitochondrial hyperpolarization. On the other hand, some literature data also indicated that those phenolic compounds that are capable of specifically inducing apoptotic cell death, e.g. resveratrol [31], act via mitochondria [32]. In any case, mitochondrial homeostasis appears as the key candidate for the target cytoplasmic activities exerted by phenolic compounds. According to the above results, apoptotic death induced by oxLDL in Caco-2 intestinal polarized cells was completely antagonized by the presence of the caspase-9 inhibitor LEHD-CHO in the culture medium while caspase-8 inhibitor IETD-CHO was ineffective. In addition,

Caco-2 cells appeared to be susceptible to Fas-induced apoptosis. However, albeit the presence of an efficient Fas-mediated pathway, oxLDL-mediated apoptotic cell death did not involve Fas receptors. In fact, Fas antagonizing antibodies were ineffective in protecting cells from oxLDL-mediated apoptosis. This can be of importance if one considers that other apoptotic stimuli provoking redox alterations such as radiation have been hypothesized to significantly involve Fas signaling [16]. Altogether these findings suggest that oxLDL induce apoptosis by directly acting on mitochondria. In particular, the apoptotic proneness of intestinal cells might be due to a peculiar mitochondrial alteration, i.e. hyperpolarization, which results in a type II cell death pathway. This also appears to be supported by the fact that by using 'baby doses' of the uncoupler agent FCCP, the inhibition of mitochondria hyperpolarization exerted by this drug also hindered oxLDL-induced apoptosis of intestinal cells. These results are in agreement with recent literature data on the antiapoptotic activity exerted by low concentrations of uncouplers [33,34]. Moreover, phenolic agents appeared devoid of uncoupler activity per se. In fact, when given alone these compounds did not modify the percentage of cells with reduced $\Delta\Psi$. By contrast, once in the presence of the uncoupler agent (FCCP), they were not only incapable of 'additive' uncoupler activity, but rather they protected intestinal cell mitochondria from $\Delta\Psi$ loss induced by FCCP. Altogether, the results herein reported clearly suggest for the first time that mitochondrial hyperpolarization, previously proposed for lymphoid cells [25], can be considered a more generalized earlier feature of apoptotic cell death, and that phenolic agents can effectively modulate intestinal cell fate.

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