British Journal of Pharmacology (1998) 123, 565–573

Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL

[1,2]Otilia Vieira, [1]Isabelle Escargueil-Blanc, [1]Olivier Meilhac, [1]Jean-Pierre Basile, [2]Joao Laranjinha, [2]Leonor Almeida, [1]Robert Salvayre & [1,3]Anne Nègre-Salvayre

[1]Department of Biochemistry, INSERM U-466, Faculty of Medicine in Rangueil, University Paul Sabatier, Toulouse, France and [2]Laboratorio de Bioquimica, Faculdade de Farmacia, Universidade de Coimbra, 3000 Coimbra, Portugal

1 Oxidized low density lipoproteins (LDL) are toxic to cultured endothelial cells. Mildly oxidized LDL, characterized by relatively low levels of TBARS and only minor modifications of apoB, were obtained by using 2 experimental model systems of oxidation, namely oxidation by u.v. radiation or ferrylmyoglobin (a two electron oxidation product from the reaction of metmyoglobin with H_2O_2).

2 Toxic concentrations of mildly oxidized LDL induce apoptosis (programmed cell death) of cultured endothelial cells, as shown by typical morphological features, by the *in situ* TUNEL procedure and by DNA fragmentation revealed on gel electrophoresis. This apoptosis is calcium-dependent and subsequent to the intense and sustained cytosolic $[Ca^{2+}]_i$ peak elicited by oxidized LDL.

3 Five naturally occurring phenolic compounds present in food and beverages were able to prevent, in a concentration-dependent manner, the apoptosis of endothelial cells induced by oxidized LDL. Among the compounds tested, caffeic acid was the most effective. Under the conditions used, the protective effect of caffeic acid (IC_{50} 8.3 ± 2.1 μmol l^{-1}) in the prevention of apoptosis induced by oxidized LDL was significantly higher than that of the other compounds tested (IC_{50}s were 12.4 ± 3.2, 14.1 ± 4.1, 20.4 ± 4.4 and 72.6 ± 9.2 μmol l^{-1} for ferulic, protocatechuic, ellagic and *p*-coumaric acids, respectively).

4 The anti-apoptotic effect of caffeic acid results from the addition of two effects, (i) the antioxidant effect which prevents LDL oxidation and subsequent toxicity ('indirect' protective effect); (ii) a 'direct' cytoprotective effect, acting at the cellular level.

5 Effective concentrations of caffeic acid acted at the cellular level by blocking the intense and sustained cytosolic $[Ca^{2+}]_i$ rise elicited by oxidized LDL.

6 In conclusion, phenolic acids (caffeic and ferulic acids being the most potent of the compounds tested under the conditions used) exhibit a potent cytoprotective effect of cultured endothelial cells against oxidized LDL. In addition to antioxidant effect delaying LDL oxidation, caffeic acid acts as a cytoprotective agent, probably by blocking the intracellular signalling triggered by oxidized LDL and culminating in the sustained calcium rise which is involved in oxidized LDL-induced apoptosis.

Keywords: Apoptosis; oxidized LDL; endothelial cells; toxicity; phenolic acids; atherogenesis

Introduction

LDL oxidation is a progressive process (Esterbauer *et al*., 1992) leading first to the formation of mildly oxidized LDL and finally to extensively oxidized LDL. Mildly oxidized LDL are characterized by a low content of lipid peroxidation products and only minor alterations of apoB, whereas extensively oxidized LDL contain high levels of lipid peroxidation products and extensively modified apoB. LDL oxidation can be promoted *in vitro* by a wide variety of oxidizing agents, such as transition metal ions (free or complexed), oxidizing enzymes, ferrylmyoglobin, living cultured cells and photonic radiations (Steinberg *et al*., 1989; Dee *et al*., 1991; Esterbauer *et al*., 1992; Nègre-Salvayre *et al*., 1992b).

Oxidized LDL are cytotoxic to cultured cells (Henricksen *et al*., 1979; Hessler *et al*., 1979). Two types of cell death, termed apoptosis and primary necrosis (or oncosis), have been discriminated on the basis of morphological studies (Willye 1981). Apoptosis is characterized by DNA fragmentation, chromatin condensation, nucleus fragmentation, organelle relocalization and cell fragmentation without increased permeability of the plasma membrane. In contrast, necrosis is characterized by cellular swelling, organelle alterations, rupture of plasma membrane and finally cell lysis and leakage of the cellular components.

During toxic cell injury, disruption of Ca^{2+} homeostasis seems to play a critical role by triggering activation of calcium-dependent degradative enzymes, resulting in irreversible damage of cellular components leading to cell death (Boobis *et al*., 1989; Orrenius *et al*., 1989; Arends *et al*., 1990; Farber, 1990). The toxic effect of oxidized LDL is mediated by an intense and sustained rise of cytosolic calcium $[Ca^{2+}]_i$ and induces a massive apoptosis of cultured endothelial cells (Nègre-Salvayre *et al*., 1992a; Escargueil-Blanc *et al*., 1997).

Dietary phenolic derivatives of cinnamic acid have been shown to prevent LDL oxidation induced by peroxyl radicals and metmyoglobin/H_2O_2 (Laranjinha *et al*., 1994; 1996). It has been proposed that these antioxidants act by quenching peroxyl radicals, reducing ferrylmyoglobin to metmyoglobin (Laranjinha *et al*., 1995) and by regenerating α-tocopherol from its radical, at the LDL surface (Laranjinha *et al*., 1995). As other naturally occuring antioxidants have been shown to protect cells against the injurious effect of oxidized LDL (Mabile *et al*., 1995), we investigated whether phenolic compounds were able to protect endothelial cells against the cytotoxic effect of oxidized LDL.

We showed here that (1) mildly oxidized LDL (oxidized by u.v. or by ferrylmyoglobin) induces a calcium-dependent

[3]Author for correspondence at: Biochimie INSERM U.466, CHU Rangueil, 1 Avenue J. Poulhès, 31403 Toulouse Cedex 04, France.

apoptosis of cultured endothelial cells; (2) caffeic acid and other phenolic compounds prevent apoptosis of endothelial cells induced by oxidized LDL; (3) beside its antioxidant effect, caffeic acid blocks the oxidized LDL induced apoptosis by inhibiting the genesis of the calcium rise induced by oxidized LDL.

Methods

Cell culture

The human umbilical vein endothelial cell line CRL-1998 was obtained from the ATCC (Rockville, MD, U.S.A.), the bovine aortic endothelial cell line GM-7372A was from the National Institute of Genetics Human Mutant Cell Repository (Camden, NJ, U.S.A.). Non immortalized bovine aortic endothelial cells (BAEC) were obtained according to the procedure of Gospodarowicz *et al*. (1976), as previously used Escargueil-Blanc *et al*., 1997). Under standard conditions, endothelial cells (0.4×10^6 cells ml^{-1}) were seeded in 6 multiwell plates, or in falcons (Nunc) when required and grown (at 5% CO_2, 37°C) in a medium supplemented with 10% heat inactivated foetal calf serum, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (CRL-1998 being grown in RPMI-1640 containing Glutamax from Gibco, GM-7273A cells and BAEC in Dulbecco's modified Eagle's DMEM medium), under previously described conditions (Escargueil-Blanc *et al*., 1997). All passages were made with a splitting ratio 1/4. Twenty four hours before LDL incorporation, this medium was replaced by a serum-free medium.

LDL isolation and oxidation

LDL from human pooled sera were isolated by ultracentrifugation according to Havel *et al*. (1986), dialyzed (against 150 mmol l^{-1} NaCl with 0.1 mmol l^{-1} EDTA), sterilized on 0.2 μm Millipore membrane and stored at 4°C under nitrogen (up to 3 weeks). LDL mobility was evaluated by electrophoresis on Hydragel and apoB by immunonephelometry.

Mildly oxidized LDL were obtained by (u.v.+copper/EDTA)-mediated oxidation under mild conditions: LDL solution (2 mg apoB/ml^{-1}, containing 5 μmol^{-1} $CuSO_4$) was irradiated for 2 h (generally), as a thin film (5 mm) in an open becher placed 10 cm under the u.v. -C source (HNS 30W OFR Osram u.v. -C tube, λ max 254 nm, 0.5 mW cm^{-2} determined by a Scientech thermopile Model 360001), under the standard conditions previously defined by Escargueil-Blanc *et al*. (1994). At the end of the irradiation, aliquots were taken up for analyses and oxidized LDL (200 μg apoB ml^{-1} under standard conditions or at the indicated concentration) were immediately incorporated in the culture medium.

Alternatively, mildly oxidized LDL were obtained by ferrylmyoglobin treatment. Ferrylmyoglobin mediated-LDL oxidation was carried out by incubation of LDL samples (2 mg apoB ml^{-1}, in PBS, pH 7.4) with 18 μM metmyoglobin/27 μM H_2O_2 during 2 h at 37°C. When LDL were oxidized in the presence of phenolic acids, they were added 3 min before the peroxide (which starts the oxidation reaction).

LDL oxidation was evaluated by determining their content of thiobarbituric acid reactive substances (TBARS) Yagi, 1987) and in lipid hydroperoxide by using the FOX-2 procedure Wolff, 1994), by the relative electrophoretic mobility (REM) on Hydragel (Sebia, Paris, Fr) and the level of trinitrobenzene sulphonic acid (TNBS)-reactive amino groups determined (Steinbrecher *et al*., 1990).

The susceptibility to oxidation of LDL from various batches was variable and some batches were more resistant to oxidation. In this case, the oxidation time was increased in order to obtain lipid peroxidation indices in the range indicated below. In the mildly oxidized LDL used here, lipid hydroperoxides ranged between 32 and 48 nmol mg^{-1} apoB, TBARS between 3.9 and 6.3 nmol mg^{-1} apoB, REM between 1.1 and 1.4, and TNBS-reactive amino groups between 91 and 97% (as % of the non oxidized LDL). In non oxidized LDL, the ranges were between 4.5 and 8.5 nmol mg^{-1} apoB for lipid hydroperoxides, 0.1 and 0.3 TBARS mg^{-1} apoB (non oxidized LDL being used as references, their REM and TNBS values were 1.0 and 100%).

Protein content was determined by the procedure of Smith *et al*. (1985).

Labelling of LDL with DiIC18 and determination of LDL uptake by endothelial cells

Purified (native) LDL or (u.v.+copper) oxLDL were fluorescently labelled with DiIC18 according to Via & Smith (1986), re-isolated by ultracentrifugation, dialysed and sterilized again as above indicated. Fluorescently-labelled LDL were added to the culture medium (at 200 μg apoB ml^{-1}, i.e. at the same concentration as for cytotoxicity experiments) and incubated with cells for 14 h. At the end of the incubation time, cells were carefully washed once with phosphate buffered saline (PBS) containing 5 g l^{-1} bovine serum albumin and once with PBS. Then cells were homogenized by sonication in 1 ml of distilled water and an aliquot was used to extract DiIC18 by the procedure of Folch *et al*. (1957) and to read the cell-associated fluorescence extracted in the chloroformic phase (by use of a Jobin-Yvon JY-3C spectrofluorometer; excitation 545 nm; emission 568 nm).

Determination of cytotoxicity and indices of necrosis and apoptosis

Subconfluent endothelial cells (grown previously under the standard conditions) were grown in a serum-free medium for 24 h, before adding lipoproteins. Then LDL or oxidized LDL were added to the culture medium at the concentration and for the time indicated below. Controls were grown under the same conditions but without addition of lipoproteins.

The whole cytotoxic effect was evaluated by using the MTT test (Denizot & Lang, 1986). Cell lysis (i.e. necrosis) was evaluated by counting trypan blue stained cells (without counting cellular debris, thus probably excluding late steps of the apoptotic and necrotic processes) (Escargueil-Blanc *et al*., 1994) and by determining the activity of lactate dehydrogenase released into the culture medium (Roche assay kit, MA kit 10), under the previously used conditions (Negre-Salvayre & Salvayre, 1992). The percentage of apoptotic cells was determined microscopically after the nucleus of the living cells had been stained with 5 μmol l^{-1} SYTO-11 (a fluorescent permeant DNA probe dissolved in dimethylsulphoxide) and immediately examined by fluorescence microscopy (Leica model Diaplan, filters for fluorescein). Alternatively, apoptotic nuclei were visualized after fixation of cells (3% paraformaldehyde for 15 min and washing in 150 mmol l^{-1} phosphate buffered saline, pH 7.4) and staining by the DNA intercalating fluorescent probe DAPI (0.1 μg ml^{-1} in pH 7.0 Tris/EDTA/NaCl, 10/10/100 mmol l^{-1}), and mounted in Fluoprep for fluorescence microscopy (Leica model Diaplan), under the previously used conditions (Escargueil-Blanc *et al*., 1994).

DNA fragmentation was visualized *in situ* on fixed cells by the TUNEL (terminal transferase-mediated dUTP-biotin nick end labelling) procedure of Gavrielli *et al*. (1992), using the terminal transferase (TdT) kit of Boehringer Mannheim (Germany). Briefly, cells grown on glass cover slides, were fixed in 3% buffered paraformaldehyde and endogenous peroxidases were inactivated by 2% H_2O_2. After rinsing, 150 μl of TdT (0.3 u μl^{-1}) and biotinylated dUTP in TdT buffer (150 mmol l^{-1} K cacodylate/25 mmol l^{-1} Tris-HCl pH 6.6, 0.25 mg ml^{-1} bovine serum albumin) and 2 mmol l^{-1} $CoCl_2$ for 1 h at 37°C. After the reaction had been stopped and the cells rinsed 4 times, the slides were covered by 150 μl of Extra-avidin Peroxidase (Sigma, MO) diluted 1/15 in water and incubated for 30 min at 37°C, washed twice and stained with 1 mg ml^{-1} DAB for 5 min at 37°C. The positive control was treated with DNAse I (1 μg ml^{-1} from Sigma, for 10 min), as indicated by Gavrielli *et al*. (1992), before being processed through the TUNEL procedure.

To evaluate the protective effect, the phenolic compounds tested (see structures in Figure 1) were added to the culture medium under conditions indicated below and the toxicity was evaluated comparatively with the control (without phenolic compounds).

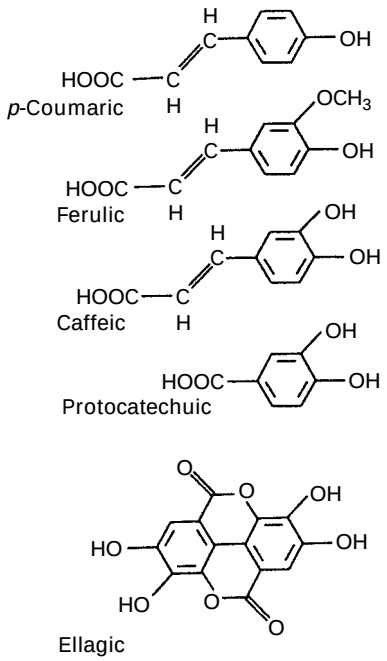

Figure 1 Chemical structure of phenolic acids.

Determination of chromatin fragments

DNA fragmentation assays were essentially derived from the procedure of McConkey *et al*. (1989) under the previously used conditions Escargueil-Blanc *et al*., 1994). Cells were allowed to lyse for 15 min in 1 ml lysis buffer (5 g l^{-1} Triton X-100 and 20 mmol l^{-1} EDTA, 5 mmol l^{-1} Tris pH 8.0), then ultracentrifuged for 20 min at 27 000 × *g* to separate the chromatin pellet from cleavage products. The pellet (re-suspended in 1 ml of 10 mmol l^{-1} Tris-HCl, pH 8.0 buffer containing 1 mmol l^{-1} EDTA) and the supernatant were assayed for DNA determination by the fluorometric DAPI procedure according to Kapuscinski & Skooczylas (1977).

Electrophoresis of DNA fragments

DNA fragmentation was visualized by gel electrophoresis (on agarose gel 1.8%, for 2 h at 50 V) under the previously used conditions Escargueil-Blanc *et al*., 1997), except that DNA bands were stained by ethidium bromide and visualized by fluorescence.

Determination of cytosolic calcium concentration $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined according to the methods of Tsien's group, by using the permeant calcium probe fura-2/AM which are hydrolyzed by intracellular carboxylesterases to liberate fura-2. Briefly, cells were incubated for 15 min at 37°C in RPMI medium buffered with 20 mmol l^{-1} HEPES and containing 0.5% bovine serum albumin and fura-2/AM (2 μmol l^{-1}). After dilution and incubation in RPMI for 45 min, cells were washed twice in phosphate buffered saline and their fluorescence recorded. $[Ca^{2+}]_i$ determination was performed at the dual excitation wavelength of 340 and 380 nm and emission at 510 nm. $[Ca^{2+}]_i$ was calculated by the ratio method according to the protocol of Thomas and Delaville (1991).

Chemicals

Dioctadecylindocarbocyanine (DiIC18) and SYTO-11 were purchased from Molecular Probes (Eugene, OR, U.S.A.), fura-2/AM, agarose, extra-avidine peroxidase, 4′,6-diamidino-2-phenylindole (DAPI), 2,4,6-trinitrobenzenesulphonic acid (TNBS), bovine serum albumin, diaminobenzidine (DAB), horse heart myoglobin and phenolic acids from Sigma (St. Louis, MO, U.S.A.), RPMI 1640 and phenol red-free (PRP-) RPMI-1640, foetal calf serum, L-glutamine, penicillin, streptomycin from Gibco (Cergy-Pontoise, France), Hydragel from Sebia (Issy, France), PGEM DNA ladder (Charbonnières, France), G-Nome kit (Bio 101, La Jolla, CA) and other chemicals from Sigma or Prolabo (Paris). Before use, metmyoglobin was purified by dialysis against phosphate buffer (20 mM phosphate, 110 mM NaCl), pH 7.4 containing 50 μM DTPA and Chelex-100. Stock metmyoglobin and H_2O_2 solutions were standardized with $\varepsilon_{632nm} = 2.1$ mM^{-1} cm^{-1} and $\varepsilon_{240nm} = 43.6$ mM^{-1} cm^{-1}, respectively. Phenolic acids solutions were prepared in water purified in a Milli-Q apparatus.

Presentation of data and statistical analysis

Data are given as means ± s.e.mean. Estimates of statistical significance was performed by ANOVA (Newman-Keuls Multiple comparison test).

Results

LDL oxidized by u.v. or by ferrylmyoglobin induced apoptosis of cultured endothelial cells

The 2 types of mildly oxidized LDL (u.v.- and FMb-oxidized LDL) used here exhibited a time- and concentration-dependent cytotoxicity to cultured endothelial cells (Figure 2a,b). CRL-1998 and GM-7273A exhibited a similar susceptibility to the toxic effect of both u.v.- and FMb-oxidized LDL, whereas non immortalized BAEC were more susceptible to the toxic effect of oxidized LDL (Table 1). In the experiments described below, we utilized the immortalized CRL-1998 cell line which was more stable and resistant than non immortalized BAEC.

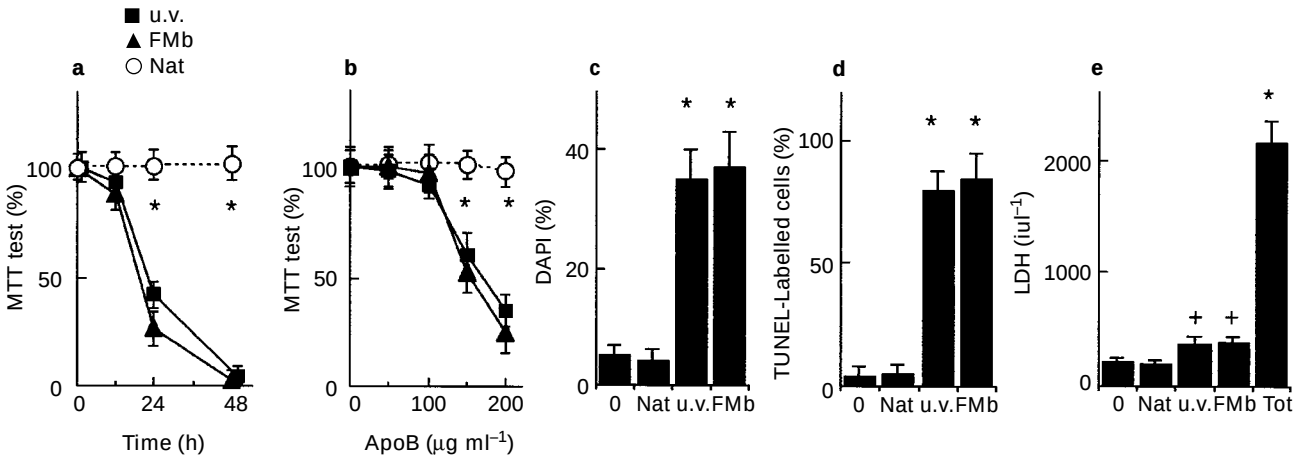

Figure 2 Toxicity of mildly oxidized LDL obtained by (u.v.+copper/EDTA) (u.v.) or by ferrylmyoglobin (FMb) treatment (described in Methods section), containing 5.2±1.4 nmol and 5.7±1.5 TBARS/mg protein, respectively. In (a) and (b), time course (with a fixed concentration of oxidized or native LDL, 200 μg apoB ml^{-1}) and concentration-dependence (CRL-1998 cells being incubated for 24 h with increasing concentrations of LDL) of the cytotoxicity. Subconfluent endothelial cells, preincubated for 24 h in serum free RPMI, were grown in the presence or absence of mildly oxLDL (solid symbols) or of native LDL (open circles, Nat). The toxic effect was evaluated by the MTT test (expressed as % of the control, i.e. cells grown in the absence of LDL). In (c, d and e), CRL-1998 cells were incubated for 24 h without (0) or with LDL (200 μg apoB ml^{-1}) either native (Nat) or oxidized by u.v.+copper/EDTA (u.v.) or by ferrylmyoglobin (FMb). In (c) and (d), DNA fragmentation was evaluated by ultracentrifugation procedure and DAPI staining, expressed as % of non sedimented chromatin (c) or by the *in situ* TUNEL procedure, expressed as labelled nuclei % cells (d). In (e), activity of LDH released in the culture medium (0, Nat, u.v., FMb) or total LDH activity (Tot) of control cell homogenate (cells grown in the absence of LDL). Mean±s.e.mean of 4 separate experiments are shown. $*P<0.01$ (comparison between controls and cells treated with u.v.- or FMb- oxidized LDL).

Table 1 Toxicity of oxidized LDL on various types of endothelial cells and protection by caffeic and ferulic acids

OxLDL (μg ml^{-1})	*Phenolic compound* (μmol l^{-1})	*u.v.-oxidized LDL*			*FMb-oxidized LDL*		
		CRL-1998	*GM-7372A*	*BAEC*	*CRL-1998*	*GM-7372A*	*BAEC*
0	0	100±8	100±9	100±10	100±8	100±9	100±10
100	0	95±7	98±9	41±7	97±8	99±8	26±4
200	0	36±5	42±4	10±4	31±6	40±6	8±3
200	100 caffeic	96±7*	94±11*	ND	93±8*	86±7*	ND
100	100 ferulic	ND	ND	ND	ND	ND	57±6*
200	100 ferulic	95±8*	92±9*	ND	91±7*	72±6*	12±4

Subconfluent endothelial cells, were treated by 200 μg apoB ml^{-1} of oxidized LDL in the absence or presence of caffeic and ferulic acids. The cytotoxicity was evaluated, at the end of the 24 h-incubation period, by the MTT test, expressed as % of the control (cells grown in the absence of oxidized LDL and phenolic compounds). Mean±s.e.mean of 3 experiments. $*P<0.01$ (comparison between assays in the absence and presence of the phenolic compound). ND, not done, because 100 μmol l^{-1} caffeic acid was toxic *per se* to BAEC (but not to CRL-1998 or to GM-7372A) and because 100 μg apoB ml^{-1} oxidized LDL were not toxic to CRL-1998 or to GM-7372A (in contrast to BAEC).

The self-toxicity of the phenolic compounds used here has been evaluated on the 3 types of endothelial cells. On CRL-1998 and GM-7273A, caffeic, ferulic, protocatechuic and coumaric acids were not toxic up to 100 μmol l^{-1} (in contrast 50 μmol l^{-1} ellagic acid was significantly toxic). In contrast, BAEC were more susceptible to the toxic effect of caffeic acid (toxicity beginning at 10 μmol l^{-1}), whereas ferulic acid was not toxic up 100 μmol l^{-1}.

Cells treated by oxidized LDL died mainly through an apoptotic process as suggested by DNA fragmentation determined by the DAPI quantitative procedure (Figure 2c), and visualized by *in situ* TUNEL labelling (Figure 2d and Figure 3). *In situ* visualization of DNA fragmentation by the TUNEL procedure is one of the earliest morphological features of apoptosis (when primary necrosis or oncosis is excluded). Under the conditions used here, *in situ* DNA fragmentation (TUNEL labelling of the nuclei) began to rise after a 12±2 h pulse with oxidized LDL, and was clearly visualized at 18 h (Figure 3). At this stage, the major part of the cell population was still attached to the culture flask and the permeability of their plasma membrane was not grossly altered, as suggested by the small number of trypan blue stained cells (data not shown). Six hours later, a large number of apoptotic cells became detached (and discarded with the medium, thus explaining the fall in the MTT index). Detached cells became rapidly permeant to trypan blue (data not shown). LDH activity released in the culture medium was significantly increased ($P<0.05$) after 24 h incubation of CRL-1998 cells with oxidized LDL (200 μg apoB ml^{-1}), but the released LDH activity was low in comparison to the total cellular LDH content (Figure 2e).

Phenolic compounds prevent oxLDS-mediated apoptosis of endothelial cells

When phenolic compounds were added to the culture medium simultaneously with u.v.-oxidized LDL (200 μg apoB ml^{-1}), the viability of endothelial cells was significantly improved in a

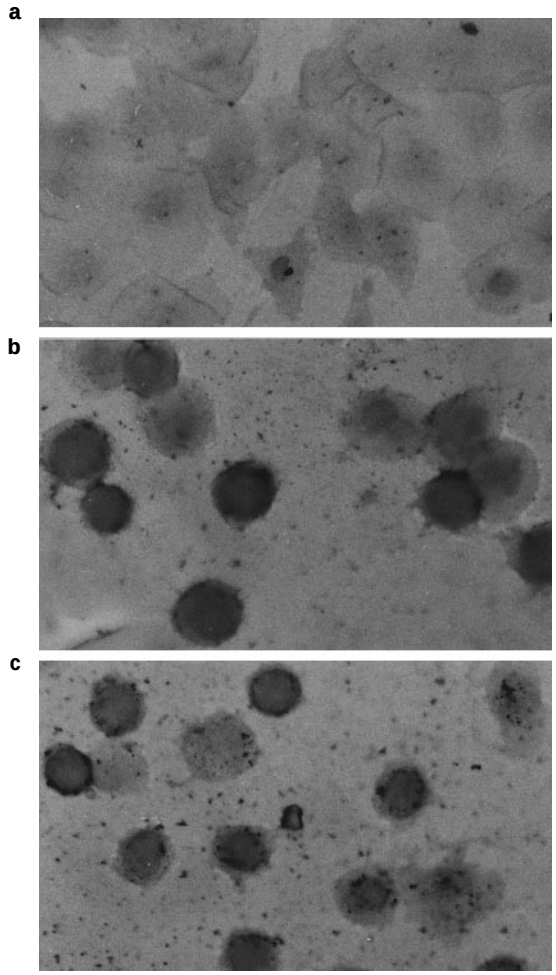

Figure 3 DNA fragmentation induced by oxidized LDL on CRL-1998 cell culture. In (a), cells grown in the absence of LDL (control). In (b) and (c), cells were grown for 24 h in the presence of a fixed concentration (200 μg apoB ml^{-1} of LDL oxidized by (u.v.+copper/EDTA) or by ferrylmyoglobin (under the same conditions as in Figure 2c,d, respectively.

concentration-dependent manner by all the compounds tested. Under the experimental conditions used in Figure 4a, caffeic acid exhibited the highest protective effect (IC_{50} 8.3 ± 2.1 μmol l^{-1}). IC_{50} of ferulic and protocatechuic acids were slightly lower (IC_{50} 12.4 ± 3.2 and 14.1 ± 4.1 μmol l^{-1}, respectively). *p*-Coumaric acid exhibited only a relatively low protective effect (IC_{50} 72.6 ± 9.2). Ellagic acid exhibited an intermediate activity (IC_{50} 20.4 ± 4.4 μmol l^{-1}), but its use was restricted by its non negligible self-toxicity (at concentrations higher than 25 μmol l^{-1}). Optimal concentrations of caffeic, protocatechuic or ferulic acids were able to inhibit apoptosis of endothelial cells induced by toxic concentrations of oxidized LDL, whereas, as expected, ellagic and *p*-coumaric acids were less effective. (Figure 4b,c).

As shown in Table 1, caffeic and ferulic acids, the most effective compounds in the protection of CRL-1998 cells, were also able to protect GM-7372A cells against the toxic effect of 200 μg apoB ml^{-1} oxidized LDL. Ferulic acid (100 μmol l^{-1}) was also able to protect BAEC against 100 μg apoB ml^{-1} oxidized LDL, but not against 200 μg apoB ml^{-1} oxidized LDL.

Phenolic compounds were also able to prevent cytotoxicity and apoptosis induced by FMb-oxidized LDL. In this experimental system, caffeic acid added to the culture medium simultaneously with oxidized LDL was able to prevent in a concentration-dependent manner cell loss and apoptosis of endothelial cells ('direct' protective effect) (Figure 5). When caffeic acid was added to the LDL preparation before oxidation, i.e. before addition of ferrylmyoglobin, the 'indirect' protective effect (antioxidant and subsequent protective effects) was significantly higher than under 'direct' conditions (addition to the culture medium with oxidized LDL, i.e. after LDL oxidation) ($P < 0.05$, comparison between 'indirect' and 'direct' conditions at 10 and 100 μmol l^{-1} (Figure 5).

When cells were preincubated for 24 h with phenolic compounds (at concentrations protecting effectively cells under co-incubation conditions), then washed and immediately incubated with toxic concentrations of oxidized LDL, all the

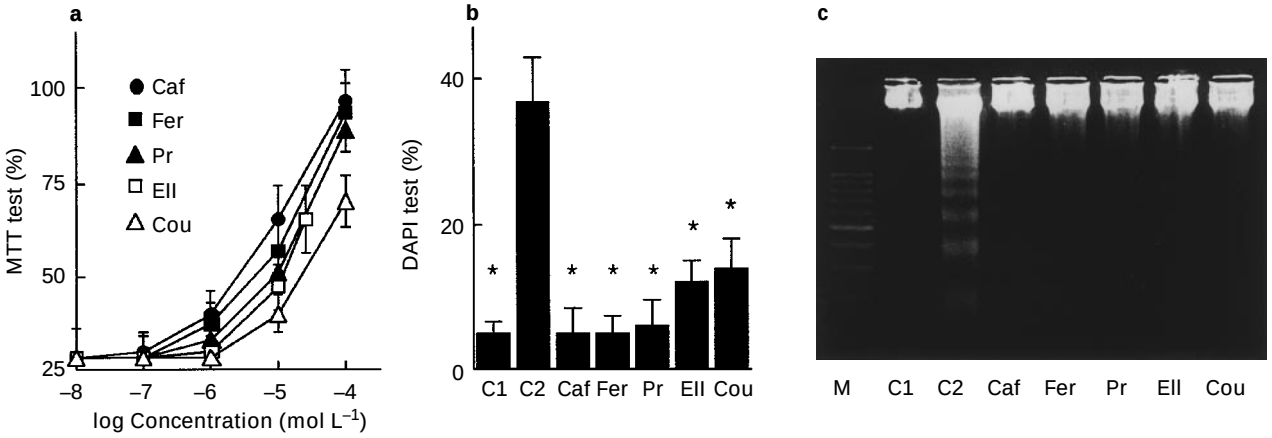

Figure 4 Protective effect of phenolic compounds against apoptosis of CRL-1998 cells induced by (u.v.+copper/EDTA)-oxidized LDL (200 μg apoB ml^{-1} for 24 h). In (a), effect of increasing concentrations of phenolic compounds. The toxic effect was evaluated by the MTT test (expressed as % of the control, i.e. cells grown in the absence of LDL). Mean $\pm$ s.e.mean of 3 separate experiments. In (b) and (c), inhibitory effect of phenolic compounds on DNA fragmentation induced by oxidized LDL. Cells were grown for 24 h either in the absence (C1) or presence of oxidized LDL without (C2) or with phenolic acids, caffeic (Caf), ferulic (Fer), protocatechuic (Pr), *p*-coumaric (Cou) and ellagic acids (Ell) (100, 100, 100, 100 and 25 μmol l^{-1}, respectively). In (b), DNA fragmentation was evaluated by the ultracentrifugation procedure (DAPI test). Mean $\pm$ s.e.mean of 3 separate experiments. $^*P < 0.01$ (comparison between controls C2 and assays containing phenolic compounds). In (c), detection of DNA fragmentation (DNA ladder) by gel electrophoresis and BET staining. M, is a PEGM DNA marker (representative experiment).

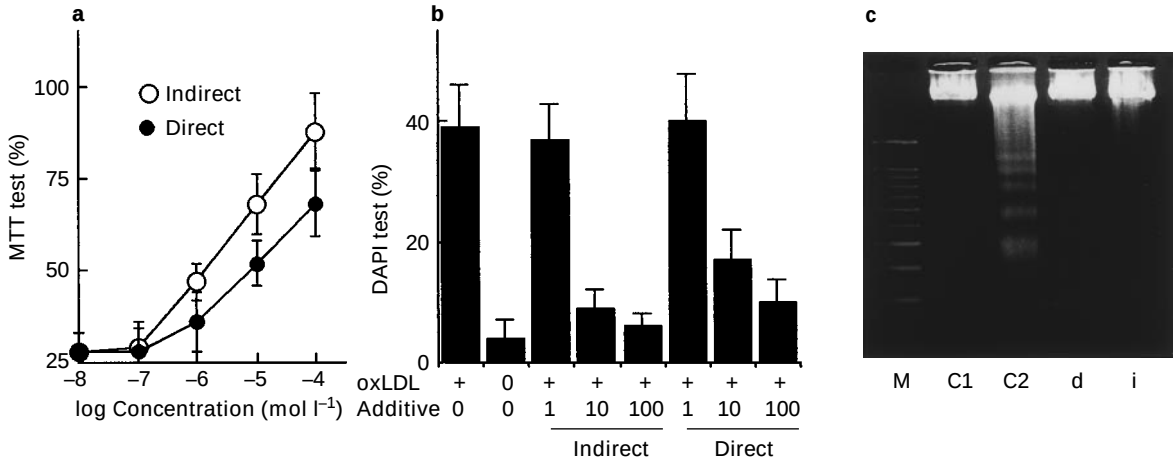

Figure 5 Protective effect of caffeic acid against the toxic effect of Fmb-oxidized LDL. Under 'indirect' conditions (i.e. evaluating both the antioxidant effect and the subsequent inhibition of the toxicity), caffeic acid (at the indicated concentration) was added to the LDL solution (2 mg apoB ml^{-1}) before oxidation and was then transferred to the cell culture (final concentration being 200 μg apoB ml^{-1}, i.e. dilution by 10 of LDL and phenaolic compounds) for testing the cytotoxicity. Under 'direct' conditions, caffeic acid (at the indicated concentration) was added to the culture medium simultaneously with 200 μg apoB ml^{-1} FMb-oxLDL (LDL were oxidized in the absence of caffeic acid). In (a), 'indirect' or 'direct' protective effect of increasing concentrations of caffeic acid. The toxicity was evaluated by the MTT test (%). In (b), inhibition of DNA fragmentation by caffeic acid (1, 10, 100 μmol l^{-1}), under 'indirect' or 'direct' conditions. In (c), gel electrophoresis showing the inhibition of DNA fragmentation by caffeic acid under 'indirect' or 'direct' conditions; controls were grown without caffeic acid and without (C1) or with FMb-oxLDL (200 μg ml^{-1}) (C2); cells were with caffeic acid (100 μmol l^{-1} under 'direct' (d) or 'indirect' (i) conditions (as in (b)). M, is a PEGM DNA marker. In (a) and (b), mean ± s.e.mean of 4 separate experiments; (c) is a representative experiment.

phenolic compounds tested were ineffective at protecting the cells (data not shown).

We also tested the protective effect of phenolic compounds added after oxidized LDL. When an effective concentration of caffeic acid (the most potent of phenolic compounds tested here) was added after oxidized LDL, endothelial cell protection was effective up to 3 h, then decreased progressively until 9 h when it reached the basal level (no protection).

Inhibition by caffeic acid of the $[Ca^{2+}]_i$ rise elicited by oxidized LDL

Under the experimental conditions used in Figure 6, $[Ca^{2+}]_i$ began to rise in endothelial cells 10 h after addition of mildly oxidized LDL, was maximal between 12–14 h and then decreased to the basal level in about 3 h. This $[Ca^{2+}]_i$ peak was concomittant with the beginning of the morphological apoptotic changes. As shown in Figure 6, effective concentrations of caffeic acid (100 μmol l^{-1}) blocked the $[Ca^{2+}]_i$ rise evoked by oxLDL. The mechanism of the protective effect of caffeic acid probably results from the inhibition of the $[Ca^{2+}]_i$ peak, since blocking the $[Ca^{2+}]_i$ rise is sufficient to block the toxic process induced by oxidized LDL.

Since the cellular uptake of oxLDL is a prerequisite for their toxic effect, the effect of phenolic compounds on the uptake of oxLDL was investigated. As shown in Table 2, the uptake of oxLDL by endothelial cells was not affected by the concentration of phenolic compounds used here. Therefore the protective effect (and the inhibition of the $[Ca^{2+}]_i$ peak) by phenolic compounds cannot be explained by the inhibition of the cellular entry of toxic compounds contained in oxidized LDL.

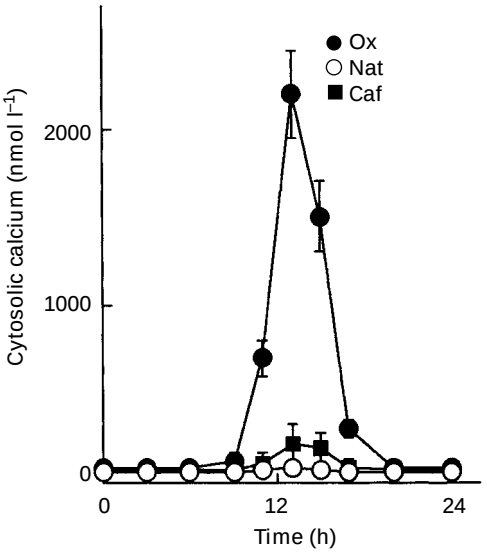

Figure 6 Time course of $[Ca^{2+}]_i$ levels in endothelial cells incubated with 200 μg apoB ml^{-1} native LDL (Nat) or with 200 μg apoB ml^{-1} (u.v. + copper/EDTA)-oxidized LDL (Ox) or with 200 μg apoB ml^{-1} (u.v. + copper/EDTA)-oxidized LDL and 100 μmol l^{-1} caffeic acid added simultaneously (Caf). $[Ca^{2+}]_i$ levels were determined fluorometrically on the whole cell population, with fura-2/AM, under conditions indicated in Methods. Mean ± s.e.mean of 3 separate experiments are shown.

Discussion

The present study showed that toxic concentrations of mildly oxLDL lead to apoptotic cell death of human cultured endothelial cells and that this apoptotic process is prevented by phenolic antioxidants, caffeic acid being the most effective of the tested compounds.

As LDL oxidation is a progressive process (Esterbauer *et al*., 1992), mildly oxidized LDL are formed during early steps of the oxidation process. Mildly oxidized LDL were obtained by methods which allow moderate oxidation to generate, i.e. treatment by (i) u.v.-C (Nègre-Salvayre *et al*., 1992a) in the

Table 2 Uptake of native (nat) or oxLDL (ox), labelled with DiIC18 and incubated with subconfluent endothelial cells for 14 h

Phenolic compound	*Concentration* (μmol l^{-1})	*DiIC18-LDL* (200 μg apoB ml^{-1})	*DiIC18-oxLDL* (200 μg apoB ml^{-1})
None	–	100 ± 6	100 ± 5
Caffeic acid	100	99 ± 7	100 ± 4
Coumaric acid	100	102 ± 7	99 ± 5
Ferulic acid	100	98 ± 8	97 ± 7
Protocatechuic acid	100	100 ± 6	99 ± 5
Ellagic acid	100	99 ± 6	101 ± 7

The nat or oxLDL were labelled with DiIC18 as indicated in Methods. After the cells had been washed and homogenized the cell-associated DiIC18 were extracted and the fluorescence determined under conditions indicated in Methods. Data are expressed as % of the control (i.e. cells grown in the presence of labelled LDL or oxLDL, but in the absence of any additive). Mean ± s.e.mean of 3 experiments.

presence of copper and EDTA (Escargueil-Blanc *et al*., 1994; 1997); (ii) ferrylmyoglobin obtained by the metmyoglobin/H_2O_2 system (Laranjinha *et al*., 1996; Dee *et al*., 1991). Mildly oxidized LDL used here were characterized by relatively low levels of lipid peroxides and TBARS, similar to those found at the end of the lag period when copper oxidation was used (Esterbauer *et al*., 1992).

Mildly oxidized LDL have been shown to be cytotoxic to endothelial cultured cells (Henricksen *et al*., 1979; Hessler *et al*., 1979), but the mechanism of this cytotoxicity remains poorly understood. Through their toxic effect on endothelial cells, oxidized LDL could take part in the endothelial cell injury (Ross, 1993). It was therefore of interest to investigate the mechanism of the toxic effect of mildly oxidized LDL on endothelial cells, and to investigate the protection of endothelial cells by dietary or pharmacological compounds.

Toxic concentrations of oxidized LDL induced apoptosis of cultured endothelial cells, as assessed by morphological and biochemical features. During the first steps of apoptosis, endothelial cells were still attached to the culture flask, but exhibited the characteristic apoptotic alterations of the nucleus, i.e. chromatin margination and condensation, nucleus fragmentation (easily visualized by fluorescent DNA probes and by electron microscopy) (Escargueil-Blanc *et al*., 1997), without an increase of plasma membrane permeability, as suggested by the lack of trypan blue staining (data not shown). The TUNEL procedure revealed that a relatively high number of cells exhibited DNA fragmention. Later on (3 – 5 h after the beginning of apoptotis), apoptotic cells lost their adherence to the culture flask, underwent plasma membrane alterations and post-apoptotic necrosis, as suggested by trypan blue staining. The level of LDH activity released by cells into the culture medium increased significantly ($P<0.05$), but remained relatively low in comparison to the total cellular LDH content. Next, these floating cells progressively disintegrated into small fragments and apoptotic bodies (data not shown). This characteristic sequence of alterations is quite consistent with the classical description of apoptosis and post-apoptotic alterations occurring *in vitro* (post-apoptotic necrosis and cell disintegration) (Willye, 1981; Golstein et al., 1991).

It should be noted that apoptosis induced by oxidized LDL is a phenomenon not restricted to the human umbilical vein endothelial immortalized cell line CRL-1998, since oxidized LDL also triggered apoptosis of the bovine aortic endothelial immortalized cell line GM-7372A and of (non immortalized) bovine aortic endothelial cells. The latter non immortalized endothelial cells were more fragile and more susceptible to a lower concentration of oxidized LDL than the immortalized endothelial cells. This study was conducted on immortalized cells because these cells were less susceptible to artefactual loss of viability and gave results generally more reliable than non immortalized cells.

Theoretically, the pathophysiological consequences of LDL oxidation can be blocked by two ways (at least): (1) by inhibiting LDL oxidation (antioxidant effect); (2) by increasing the resistance of cells against the toxic effect of lipid peroxidation products contained in oxidized LDL (direct cytoprotective effect).

The antioxidant activity of phenolic compounds and their ability to prevent LDL oxidation, has been recently documented by Laranjinha *et al*. (1996). As expected, when LDL oxidation was inhibited by effective concentrations of phenolic compounds, the subsequent toxicity was also prevented. This 'indirect' protective effect is probably due to the inhibition of formation of lipid peroxidation derivatives (Laranjinha *et al*., 1996) which are known to be cytotoxic (Nègre-Salvayre *et al*., 1990; Colles *et al*., 1996).

Moreover, phenolic compounds also exhibited a 'direct' protective effect of endothelial cells against apoptosis induced by oxidized LDL (independently of the inhibition of LDL oxidation). These 'direct' anti-apoptotic effects were clearly independent of the inhibition of LDL oxidation (i.e. 'indirect' protection due to the antioxidant effect), since, in experiments testing the 'direct' protective effect, LDL were (pre-)oxidized in the absence of phenolic compounds and phenolic compounds were added to the culture medium simultaneously with oxidized LDL (i.e. after LDL oxidation). This 'direct' protective effect is consistent with previous observations with other compounds (Négre-Salvayre & Salvayre, 1992; Mabile *et al*. 1995; Schmitt *et al*., 1995; Colles *et al*. 1996).

It should be noted that, under the conditions used here, the 'indirect' protective effect of caffeic acid (resulting from its antioxidant effect) was significantly higher than the 'direct' cytoprotective effect. This is in contrast with a-tocopherol which is much more effective under 'direct' protective conditions than under 'indirect conditions' (Mabile *et al*. 1995).

The antiapoptotic effect was not persistent, when cells, preincubated with phenolic compounds, were incubated with oxidized LDL after the phenolic compounds had been washed out, they behaved as before. These results are completely different from those observed with α-tocopherol which exhibited a persistent protective effect for several days (Mabile *et al*, 1995). This discrepancy may be due to different mechanisms of action at the cellular level or to a more rapid extrusion or metabolic inactivation of phenolic compounds.

The precise mechanism of the apoptotic effect of oxidized LDL remains largely unknown, therefore the antiapoptotic effect of phenolic compounds is also unknown. The data presented here show that caffeic acid blocks the intracellular signalling triggered by oxidized LDL. The sustained $[Ca^{2+}]_i$ peak is involved in the apoptotic process induced by oxidized LDL as suggested by the time course of the $[Ca^{2+}]_i$ peak and cytotoxicity ($[Ca^{2+}]_i$ peak occurring before the cytotoxicity) and by the protective effect of agents (i.e. the calcium chelator EGTA and the calcium channel blocker nifedipine) which block concomitantly the $[Ca^{2+}]_i$ peak and the apoptotic process as well (Escargueil-Blanc *et al*., 1994; 1997). In endothelial cells, the $[Ca^{2+}]_i$ rise elicited by oxidized LDL induces very probably the activation of calcium-dependent endonucleases involved in the apoptotic process (as suggested

by the internucleosomal DNA cleavage and its inhibition by aurintricarboxylic acid) (Escargueil-Blanc *et al*. 1997). These data are in good agreement with previous studies on the role of calcium in the lethal cell injury (Orrenius *et al*. 1989; Farber 1990).

As effective concentrations of caffeic acid blocked concomitantly the $[Ca^{2+}]_i$ rise and apoptosis evoked by oxidized LDL, it is suggested that the antiapoptotic effect of caffeic acid may result from its ability to inhibit the intracellular signalling cascade (yet unidentified) triggered by oxidized LDL and culminating in the sustained $[Ca^{2+}]_i$ peak. On the other hand, another possible mechanism for the antiapoptotic effect of caffeic acid may involve the potential regeneration of α-tocopherol from its radical in cell membranes, since phenolic compounds are able to regenerate a-tocopherol at the LDL surface (Laranjinha *et al*. 1995) and since α-tocopherol is able to protect cells against the toxic effect of oxidized LDL (Mabile *et al*. 1995; Schmitt *et al*. 1995).

From a pathophysiological point of view, the endothelial cell injury elicited by oxidized LDL may be involved in atherogenesis and thrombosis (Witztum & Steinberg, 1991; Ross 1993). The endothelial defects are associated with platelet adhesion and thrombi formation and are potentially involved in extensive thrombotic events. One of the most striking results of the present study is the efficiency of caffeic, ferulic and protocatechuic acids and other phenolic acids in protecting endothelial cells against apoptosis induced by oxidized LDL. Finally, since phenolic acids are present *in vivo* in human plasma (Goldstein *et al*., 1984), it may be speculated that they could take part in maintaining the integrity of the endothelial cell lining by preventing LDL oxidation [Laranjinha et al., 1996) and by increasing the resistance of endothelial cells against injurious effect of oxidized LDL.

The authors wish to thank Dr A. Parini for statistical advice, J.C. Thiers for iconography and C. Mora for the technical assistance. This work was supported by grants from INSERM, Conseil Régional Midi-Pyrénées, MESR and University Toulouse-3. Support for O. Vieira was provided through a grant from the PRAXIS XXI (BD/5493/95).

References

ARENDS, M.J., MORRIS, R.G. & WILLYE, A.H. (1990). Apoptosis: the role of the endonuclease. *Am. J. Pathol.,* **136,** 593 – 608.

BOOBIS, A.R., FAWTHROP, D.J. & DAVIES, D.S. (1989). Mechanisms of cell death. *Trends Pharmacol. Sci.,* **10,** 275 – 280.

COLLES, S.M., IRWIN, K.C. & CHISOLM, G.M. (1996). Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7 beta-hydroperoxycholesterol. *J. Lipid Res.,* **37,** 2018 – 2028.

DEE, G., RICE-EVANS, C., OBEYESEKERA, S., MERAJI, S., JACOBS, M. & BRUCKDORFER, K.R. (1991). The modulation of ferrylmyoglobin formation and its oxidative effects on LDL by nitric oxide. *FEBS Lett.,* **294,** 38 – 42.

DENIZOT, F. & LANG, R. (1986). Rapid colorimetric assay for cell growth and survival: modifications to tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Meth.,* **89,** 271 – 277.

ESCARGUEIL-BLANC, I., MEILHAC, O., PIERAGGI, M.T., ARNAL, J.F., SALVAYRE, R. & NÈGRE-SALVAYRE, A. (1997). Oxidized LDL induce massive apoptosis of cultured human endothelial cells through a calcium-dependent pathway. Prevention by aurintricarboxylic acid. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 331 – 339.

ESCARGUEIL-BLANC, I., SALVAYRE, R. & NÈGRE-SALVAYRE, A. (1994). Necrosis and apoptosis induced by oxidized LDL occurs through two calcium-dependent pathways in lymphoblastoid cells. *FASEB J.,* **8,** 1075 – 1080.

ESTERBAUER, H., DIEBER-ROTHENEDER, M., WAEG, G., STRIEGL, G. & JÜRGENS, G. (1990). Biochemical, structural and functional properties of oxidized LDL. *Chem. Res. Toxicol.,* **3,** 77 – 92.

ESTERBAUER, H., GEBICKI, J., PUHL, H. & JÜRGENS, G. (1992). The role of lipid peroxidation and oxidative modification of LDL. *Free. Rad. Biol. Med.,* **13,** 341 – 390.

FARBER, J.L. (1990). The role of calcium in lethal cell injury. *Chem. Res. Toxicol.,* **3,** 503 – 508.

FOLCH, J., LEES, M. & SLOANE STANLEY, G.N. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.,* **226,** 497 – 509.

GAVRIELLI, Y., SHERMAN, Y. & BEN-SASSON, S.A. (1992). Identification of programmed cell death in situ via a specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.,* **119,** 493 – 503.

GOLDSTEIN, D., STULL, R., MARKY, S., MARKS, E. & KEISER, H. (1984). Dihydrocaffeic acid: a common contaminant in the liquid chromatographic-electrochemical measurement of plasma catecholamines in man. *J. Chromatogr.,* **311,** 148 – 153.

GOLSTEIN, P., OJCIUS, D.M. & YOUNG, J.D.E. (1991). Cell death mechanisms and the immune system. *Immunol. Rev.,* **121,** 29 – 65.

GOSPODAROWICZ, D., MORAN, J., BRAUN, D. & BIRDWELL, C.R. (1976). Clonal growth of bovine endothelial cells in tissue culture: FGF as survival agent. *Proc. Natl. Acad. Sci. U.S.A.,* **73,** 4120 – 4124.

HAVEL, R.I., EDER, H.A. & BRAIGON, J.H. (1986). The distribution and chemical composition of ultracentrifugally seperated lipoproteins in human serum. *J. Clin. Invest.,* **39,** 1345 – 1363.

HENRICKSEN, T., EVENSEN, S.A. & CARLANDER, B. (1979). Injury to human endothelial cells in culture induced by LDL. *Scand. J. Clin. Lab. Invest.,* **39,** 361 – 368.

HESSLER, J.L., ROBERTSON, A.L. & CHISOLM, G.M. (1979). LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis,* **32,** 213 – 229.

KAPUSCINSKI, J. & SKOOCZYLAS, B. (1977). Simple and rapid fluorometric method for DNA microassay. *Anal. Biochem.,* **83,** 252 – 257.

LARANJINHA, J., ALMEIDA, L. & MADEIRA, V. (1992). Lipid peroxidation and its inhibition in low density lipoproteins: quenching of cis-parinaric acid fluorescence. *Arch. Biochem. Biophys.,* **297,** 147 – 154.

LARANJINHA, J., ALMEIDA, L. & MADEIRA, V. (1994). Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low density lipoprotein peroxidation. *Biochem Pharmacol.,* **48,** 487 – 494.

LARANJINHA, J., ALMEIDA, L. & MADEIRA, V. (1995). Reduction of ferrylmyoglobin by dietary phenolic acid derivatives of cinnamic acid. *Free Rad. Biol. Med.,* **19,** 329 – 337.

LARANJINHA, J., VIEIRA, O., ALMEIDA, L. & MADEIRA, V. (1996). Inhibition of metmyoglobin/ H_2O_2-dependent LDL lipid peroxidation by naturally occuring phenolic acids. *Biochem. Pharmacol.,* **51,** 395 – 402.

MABILE, L., FITOUSSI, G., PERIQUET, B., SCHMITT, A., SALVAYRE, R. & NEGRE-SALVAYRE, A. (1995). α-Tocopherol and Trolox block the early intracellular events, TBARS and calcium rise elicited by oxidized low density lipoproteins in cultured endothelial cells. *Free Rad. Biol. Med.,* **19,** 177 – 187.

MCCONKEY, D.J., HARTZELL, P., NICOTERA, & ORRENIUS, S. (1989). Calcium-activated DNA fragmentation kills immature thymocytes. *FASEB J.,* **3,** 1843 – 1849.

NÉGRE-SALVAYRE, A., FITOUSSI, G., RÉAUD, V., PIERRAGGI, M.T., THIERS, J.C. & SALVAYRE, R. (1992a). A delayed and sustained rise of cytosolic calcium is elicited by oxidized LDL in cultured bovine endothelial cells. *FEBS Lett.,* **299,** 60 – 65.

NÉGRE-SALVAYRE, A., LOPEZ, M., LEVADE, T., PIERAGGI, M.T., DOUSSET, N., DOUSTE-BLAZY, L. & SALVAYRE, R. (1990). Ultraviolet-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. II. Uptake and cytotoxicity of ultraviolet-treated LDL on lymphoid cell lines. *Biochim. Biophys. Acta,* **1045,** 224 – 232.

NÉGRE-SALVAYRE, A., PAILLOUS, N., DOUSSET, N., BASCOUL, J. & SALVAYRE, R. (1992b). Wavelength dependence of photoinduced peroxidation and cytotoxicity of LDL. *Photochem. Photobiol.,* **55,** 197 – 204.

NÉGRE-SALVAYRE, A. & SALVAYRE, R. (1992). Protection by calcium channel blockers (nifedipine, diltiazem, and verapamil) against the toxicity of oxidized LDL to cultured lymphoid cells. *Br. J. Pharmacol.,* **107,** 738–744.

ORRENIUS, S., MCCONKEY, D.J., BELLOMO, G. & NICOTERA, P. (1989). Role of calcium in toxic cell killing. *Trends Pharmacol. Sci.,* **10,** 281–285.

ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* **362,** 801–809.

SMITH, P.K., KHRON, R.I., HERMANSON, G.T., MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J. & KLENK, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.,* **150,** 76–85.

SCHMITT, A., NEGRE-SALVAYRE, A., DELCHAMBRE, J. & SALVAYRE, R. (1995). Prevention by α-tocopherol and rutin of glutathione and ATP depletion induced by oxidized LDL in cultured cells. *Br. J. Pharmacol.,* **116,** 1985–1990.

STEINBERG, D., PARTHASARATHY, S., CAREW, T., KHOO, J.C. & WITZUM, J.L.N. (1989). Beyond cholesterol. Modification of LDL that increase its atherogenicity. *N. Engl. J. Med.,* **320,** 915–924.

STEINBRECHER, U.P., ZHANG, H. & LOUGHEED, M. (1990). Role of oxidatively modified LDL in atherosclerosis. *Free. Rad. Biol. Med.,* **9,** 155–168.

THOMAS, A.P. & DELAVILLE, F. (1991). The use of fluorescent indicators for measurement of cytosolic free calcium concentration in cell populations and single cell. In *Cellular Calcium, a Practical Approach* ed. McCormack, J.G. & Cobbold, P.H. pp. 1–54. New York: RL Press.

VIA, D.P. & SMITH, L.C. (1986). Fluorescent labelling of lipoproteins. *Meth. Enzymol.,* **129,** 848–857.

WILLYE, A.H. (1981). Cell death: a new classification separating apoptosis from necrosis. In *Cell Death in Biology and Pathology,* ed. Bowen, I.D. & Lockshin, R.A., pp. 9–34, London and New York: Chapman & Hall.

WITZUM, J.L. & STEINBERG, D. (1991). Role of oxidized LDL in atherogenesis. *J. Clin. Invest.,* **88,** 1785–1792.

WOLFF, S.P. (1994). Ferrous ion oxidation in the presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Meth. Enzymol.,* **233,** 182–189.

YAGI, K. (1987). Lipid peroxides and human diseases. *Chem. Phys. Lipids,* **45,** 337–351.

(Received March 26, 1997
Revised September 29, 1997
Accepted October 20, 1997)