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Prevention by α-tocopherol and rutin of glutathione and ATP depletion induced by oxidized LDL in cultured endothelial cells

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1 Oxidized low density lipoproteins (LDL) are thought to play an important role in atherogenesis. Mildly oxidized LDL are cytotoxic to cultured endothelial cells. Toxic doses of oxidized LDL promote the peroxidation of cellular lipids (beginning at 6 h and being maximal after 12 h of pulse with oxidized LDL) and glutathione and ATP depletion (beginning after 15 h of pulse and evolving concurrently with the cytotoxicity).

2 Antioxidants from 3 different classes (rutin, ascorbic acid and α-tocopherol) were compared as to their ability to inhibit the cytotoxic effect of oxidized LDL to endothelial cells.

3 Effective concentrations of α-tocopherol inhibited cellular lipid peroxidation, glutathione and ATP depletion and the cytotoxic effect.

4 Ascorbic acid was less effective than α-tocopherol and rutin, and exhibited a dose-dependent biphasic effect in the presence of oxidized LDL.

5 Effective concentrations of rutin inhibited glutathione and ATP depletion as well as cytotoxicity, but did not block cellular lipid peroxidation. This suggests that the glutathione and ATP depletion is directly correlated to the cytotoxicity of oxidized LDL, whereas cellular lipid peroxidation is probably not directly the cause of cellular damage leading to cell death.

6 The association of antioxidants of 3 different classes allowed the suppression of the biphasic effect of ascorbic acid and increased the efficacy of the protective effect. The potential consequences for prevention of the pathogenic role of oxidized LDL in endothelial injury are discussed.

Keywords: Low density lipoproteins; oxidized LDL; cytotoxicity; endothelial cells; rutin; α-tocopherol; ascorbic acid; ATP-glutathione

Introduction

Low density lipoproteins (LDL) play an important role in atherogenesis as suggested by experimental and clinical studies on Familial Hypercholesterolaemia and by epidemiological studies on hyperlipidaemias and coronary heart diseases (Goldstein & Brown 1977; 1989). Oxidized LDL are thought to play a central role in the formation of macrophagic 'foam cells' (characteristic of early lesions of atheroma), by inducing deviation of oxidized LDL towards the scavenger-receptor pathway of macrophagic cells, recruitment of monocytes and inhibition of the motility of resident macrophages (Steinberg *et al.*, 1989; Witztum & Steinberg 1991). As oxidized LDL are cytotoxic to cultured cells (Henricksen *et al.*, 1979; Hessler *et al.*, 1979), it may be speculated that they could be involved in endothelial cell injury and subsequently in the atherogenic process (Ross, 1986).

LDL can be oxidized *in vitro* by cultured vascular cells and by transition metals (see reviews by Esterbauer *et al.*, 1990; Steinbrecher *et al.*, 1990). Mildly oxidized LDL (LDL containing lipid peroxidation derivatives with only minor alterations of apoB) can be obtained by u.v. irradiation (Nègre-Salvayre *et al.*, 1990; Salvayre *et al.*, 1990) or by u.v. irradiation in the presence of copper ions (Escargueil-Blanc *et al.*, 1994). When taken up by cells, mildly oxidized LDL induce a rise of cellular thiobarbituric reactive substances (TBARS) (Nègre-Salvayre & Salvayre, 1992), which are generally considered as the hallmark of lipid peroxidation (Halliwell & Gutteridge, 1989). Oxidized LDL also induce a depletion of cellular glutathione (Kuzuya *et al.*, 1989) which may be the consequence of oxidative stress (Boobis *et al.*, 1989). Such an oxidation process may theoretically be inhibited by increasing the antioxidant defence systems of the cell (Thomas *et al.*, 1993). Antioxidants are able to protect cells against the cytotoxic effect of oxidized LDL (Nègre-Salvayre *et al.*, 1991a,b) by at least two mechanisms: (i) extracellular inhibition of LDL oxidation; (ii) 'direct' protection of cells by increase of cellular resistance against the toxicity of oxidized LDL.

α-Tocopherol is the major natural lipophilic antioxidant preventing LDL oxidation (Jessup *et al.*, 1990; Esterbauer *et al.*, 1992) and lipid peroxidation of the cell membrane by trapping peroxyl radicals and by breaking the lipid peroxidation cycle reaction (Halliwell & Gutteridge, 1989). Ascorbic acid is a superoxide radical anion scavenger (Bendich *et al.*, 1986) and the most effective aqueous-phase antioxidant in human blood plasma (Frei *et al.*, 1989). α-Tocopherol and ascorbic acid are able to cooperate in order to suppress the peroxidation of membrane phospholipids (Scarpa *et al.*, 1984). Flavonoids (derivatives of phenylchromone ring), a large group of compounds naturally occurring in higher and lower plants, have been shown to affect various biological functions, such as capillary permeability and inflammatory response (Middleton, 1984). Some flavonoids are oxygen radical scavengers (Robak & Griglewski, 1988) and good metal chelators effective in preventing lipid peroxidation (Afanas'ev *et al.*, 1989) and LDL oxidation (De Whalley *et al.*, 1990; Nègre-Salvayre *et al.*, 1991a,b; Nègre-Salvayre & Salvayre, 1992).

We have recently reported that quercetin and rutin have a direct cytoprotective effect at the cellular level against the toxicity of oxidized LDL (Nègre-Salvayre & Salvayre, 1992). The mechanism of the cellular protection induced by these antioxidants is almost completely unknown. This led us to investigate and to compare the roles of α-tocopherol, ascorbic acid and rutin in preventing the cellular oxidative stress induced by oxidized LDL.

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We found that the 3 antioxidants used here exhibited different patterns of cellular protection: (i) α-tocopherol was able to inhibit the cytotoxic effect, the rise in TBARS as well as the glutathione and ATP depletion; (ii) ascorbic acid was poorly effective when used alone; (iii) rutin was able to inhibit the depletion of glutathione and ATP and the cytotoxic effect, but did not block the rise in TBARS. We conclude that the glutathione and ATP depletion is directly correlated to the cytotoxicity of oxidized LDL, in contrast to cellular TBARS. The potential consequences for preventing vascular diseases are discussed.

Methods

Culture of bovine endothelial cells

The bovine endothelial cells (GM 7372A from the NIGM human genetic mutant cell repository, Camden, NJ, U.S.A.) were seeded in 24 or 96 multiwell culture plates (Nunc) and grown in RPMI 1640 medium containing 10% foetal calf serum, penicillin, streptomycin and glutamine; 48 h before LDL incorporation, this medium was removed and replaced by RPMI 1640 containing 2% Ultroser G (a lipoprotein-free serum substitute) as previously used (Nègre-Salvayre & Salvayre, 1992).

LDL isolation and oxidation

LDL from human pooled sera were isolated by sequential ultracentrifugation according to Havel *et al.* (1955), dialyzed, sterilized by filtration (0.2 μm Millipore membrane), their purity controlled as previously described (Nègre-Salvayre *et al.*, 1990; Nègre-Salvayre & Salvayre, 1992) and stored at 4°C under N_2 (up to 2 weeks).

LDL oxidation was promoted by u.v. irradiation (500 μW cm^{-2} for 2 h) in the presence of 2 μmol l^{-1} $CuSO_4$ (Escargueil-Blanc *et al.*, 1994) leading to the formation of mildly oxidized LDL (containing between 4–6 nmol TBARS mg^{-1} apoB). Oxidized LDL were immediately incorporated into the culture medium. TBARS were determined according to Yagi (1987).

Labelling of LDL with [^{3}H]-cholesteryl oleyl ether and determination of LDL uptake

LDL were labelled with [^{3}H]-cholesteryl oleyl ether (10^5d.p.m. mg^{-1} apoB) according to the procedure of Roberts *et al.* (1985), isolated again by ultracentrifugation, dialyzed and incorporated into the cell culture medium under the previously used conditions (Nègre-Salvayre *et al.*, 1990). The cellular uptake of radiolabelled LDL was evaluated as previously indicated (Nègre-Salvayre *et al.*, 1990); briefly, cells were incubated for 12 h with radiolabelled oxidized LDL (with [^{3}H]-cholesteryl oleyl ether) and with or without the tested molecules (α-tocopherol, ascorbic acid and rutin). At the end of the incubation, cells were washed twice in phosphate-buffered saline containing 0.5% bovine serum albumin. After scraping off the cells, the cell-associated radioactivity was counted in a liquid scintillation counter (Packard-model Tricarb 2100).

Determination of cytotoxicity and cytoprotective effect of antioxidants

In continuous pulse experiments, cells were grown in the presence of oxidized LDL until the cell viability was determined at the indicated time (48 h-pulse period, under standard conditions). Cells were washed twice with phosphate-buffered saline and the cell viability was determined by the MTT test (Price & McMillan, 1989); briefly, cells were incubated with MTT dissolved in phenol red-free RPMI (250 μg ml^{-1}), for 2 h at 37°C. Then this incubation medium was discarded and after addition of 500 μl dimethylsulphoxide (DMSO) the optical density was immediately measured (at 540 nm, with a Titertek spectrophotometer). The results were expressed as percentage of control (cells grown in the absence of oxidized LDL). Alternatively the cytotoxicity was evaluated by determining the LDH release by using a BioMerieux kit (Nègre-Salvayre *et al.*, 1990).

In order to evaluate the remaining protective effect, cells were incubated with a fixed concentration of these compounds for 48 h, washed twice and grown in fresh culture medium (free of any additional antoxidant) for variable intervals of time (up to 5 days). Then oxidized LDL were added to the culture medium and the cytotoxicity was evaluated 2 days later by the MTT test.

Determination of the cellular content of glutathione

The cellular glutathione was determined fluorometrically with monochlorobimane which is able to form glutathione-bimane adducts in the presence of glutathione S-transferase. The determination of the glutathione content was performed according to the procedures of Rice *et al.* (1986) modified by Fernandez-Checa & Kaplowitz (1990).

Determination of cellular levels of ATP

The cellular ATP levels were determined by the luciferin/luciferase system using an ATP assay kit (Calbiochem-Behring) and a LKB luminometer (model 1250 M).

Determination of thiobarbituric acid reactive substances (TBARS) and proteins

Lipid peroxidation in LDL or cell homogenates was evaluated by determining the content of thiobarbituric acid reactive substances (TBARS) according to the method of Yagi (1987) under the conditions used previously (Nègre-Salvayre *et al.*, 1990; Nègre-Salvayre & Salvayre, 1992).

Protein concentration was determined by the method of Lowry *et al.* (1951).

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), α-tocopherol, ascorbic acid, rutin and catechin were purchased from Sigma (St Louis, MO, U.S.A.), monochlorobimane from Lambda (Graz, Austria), [^{3}H]-cholesteryl oleyl ether (38 Ci $mmol^{-1}$) from Amersham, 1,1,3,3-tetraethoxypropane from Fluka (Buchs, Switzerland), RPMI 1640, foetal calf serum, penicillin, streptomycin, phosphate buffered saline from Gibco (Paris, France), Ultroser G from IBF (Villeneuve-la-Garenne, France), and the other chemicals from Merck (Darmstadt, Germany) or Prolabo (Paris, France).

Results

Time course of TBARS formation, depletion of glutathione and ATP and cytotoxicity in endothelial cells treated with mildly oxidized LDL

As shown in Figure 1, toxic doses of oxidized LDL (200 μg apoB ml^{-1}) induced the formation of cellular TBARS which began to rise after 6 h of the pulse and culminated 6 h later (Figure 1a). The cellular level of glutathione exhibited a biphasic variation: a transient but significant increase (+40 to 50%) occurred between 15–20 h and was then followed by a progressive decrease (Figure 1b). The level of cellular ATP did not change significantly during the first 10 h and declined progressively after 15 h (Figure 1c). The cytotoxic indices (MTT test) (Figure 1d) began to rise after 15 h and was markedly correlated to ATP depletion. In the same period of time, native (non oxidized) LDL did not induce any significant

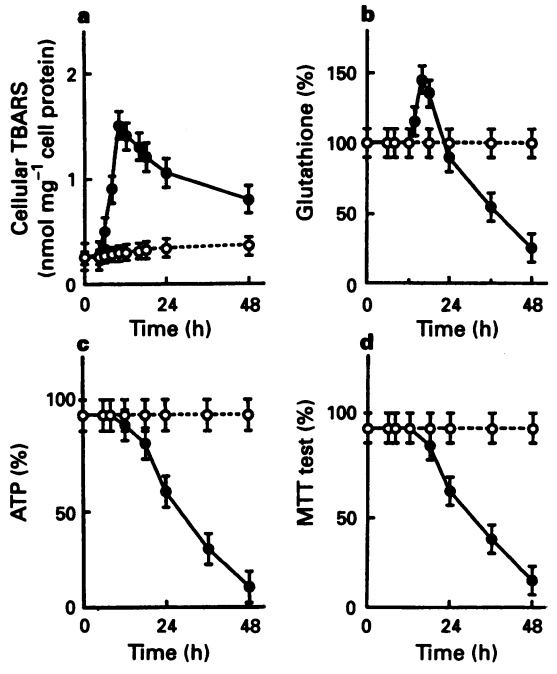

Figure 1 Time course of cellular oxidative stress indices, i.e. TBARS (a), glutathione (b), ATP (c) and cytotoxicity (d) induced by a fixed dose (200 μg apoB ml^{-1}) of native (○) or mildly oxidized LDL (●). LDL oxidation was performed under the standard conditions indicated in the Methods section; mildly oxidized LDL contained 6±1.5 nmol TBARS mg^{-1} apoB. Mean±s.e.mean of 3 separate experiments performed with the same batch of LDL.

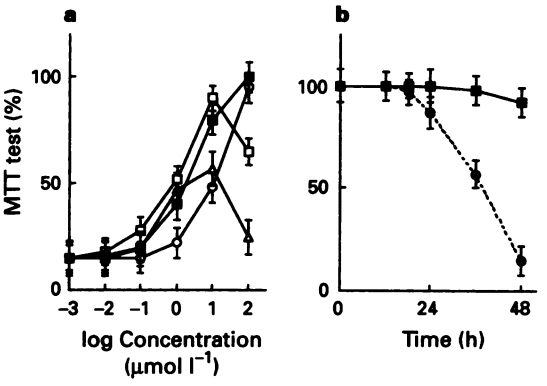

Figure 2 Cytoprotective effect of antioxidants added to the culture medium simultaneously with a fixed dose of oxidized LDL (200 μg apoB ml^{-1}. In (a), direct cytoprotective effect of increasing concentrations of ascorbic acid (△), α-tocopherol (○) and rutin (□) and of the mixture rutin/ascorbic acid/α-tocopherol, 4/4/1 (by mol, expressed as rutin concentration) (■) against the cytotoxicity of a fixed concentration of oxidized LDL (LDL oxidized under the standard conditions in the absence of any additional antioxidant). Increasing doses of antioxidants were added to the culture medium simultaneously with oxidized LDL (200 μg apoB ml^{-1}), and after 48 h incubation the cytotoxic effect was evaluated by the MTT test, expressed as % of the control cells grown in the absence of oxidized LDL or antioxidant. Mean±s.e.mean of 3 separate experiments performed with the same batch of LDL. In (b), time course of the cytotoxicity of oxidized LDL (●) and protection by the mixture rutin/ascorbic acid/α-tocopherol (■). Mean±s.e.mean of 3 experiments (each point being done in duplicate).

Table 1 Self-cytotoxicity of increasing concentrations of antioxidants to cultured endothelial cells (after incubation of cells for 48h with the indicated concentrations of antioxidants). The cytotoxicity was evaluated by the MTT test and expressed as % of control.

	Antioxidant concentration (μmol l^{-1})			
	0	1	10^1	10^2
Rutin	100±6	100±7	95±6	70±5
Ascorbic acid	100±5	100±7	100±4	95±5
α-Tocopherol	100±7	100±5	100±7	100±4
Rutin + ascorbic acid + α-tocopherol*	100±7	100±3	100±5	95±4

Means±s.e.mean of 3 experiments using the same pool of LDL (each point being done in duplicate).
* Rutin/ascorbic acid/α-tocopherol, 4/4/1 (by mol), concentration expressed as rutin or ascorbic acid concentration.

variation of the tested cellular parameters (TBARS, glutathione, ATP, cytotoxicity).

Protection of endothelial cells by α-tocopherol, ascorbic acid and rutin against the cytotoxic effect of oxidized LDL

In order to test the potential cytoprotective effect of antioxidants, increasing concentrations of the tested antioxidants (α-tocopherol, ascorbic acid and rutin) were added to the culture medium simultaneously with a toxic dose (200 μg apoB ml^{-1}) of oxidized LDL (LDL previously oxidized in the absence of any additive). As shown in Figure 2a, a relatively high concentration of α-tocopherol (100 μmol l^{-1}) was able to protect the cells almost completely. Ascorbic acid and rutin exhibited a potent protective effect, but did not protect the cells completely because of a biphasic effect observed at high concentrations (higher than 1 and 10 μmol l^{-1} for ascorbic acid and rutin, respectively). In order to investigate the mechanism of the biphasic effect, we tested the self-cytotoxicity of ascorbic acid and rutin in the absence of oxidized LDL (Table 1). Rutin exhibited a moderate cytotoxic effect at 100 μmol l^{-1}, whereas α-tocopherol and ascorbic acid did not show any significant cytotoxicity over the concentration range employed here (up to 100 μmol l^{-1}). High doses of ascorbic acid were not cytotoxic *per se*, but only in the presence of oxidized LDL. This effect could be due to the biphasic redox properties of ascorbic acid (antioxidant at low concentrations and pro-oxidant at high concentrations) (Wefers & Sies, 1988). Since the pro-oxidant effect can be shifted to the antioxidant side by the association with α-tocopherol (Wefers & Sies, 1988) and since the mixture of the 3 compounds exhibited an additive antioxidant effect (Nègre-Salvayre *et al.*, 1991b) we tried to obtain a better cytoprotective effect by associating the 3 antioxidants. As expected, this association (in the molar ratio rutin/ascorbic acid/α-tocopherol, 4/4/1) exhibited a strong protective effect on endothelial cells.

In order to exclude the possibility that the protective effect could result from the interaction between antioxidant and oxidized LDL (resulting in the inactivation of oxidized LDL in the culture medium), we performed pre-incubation experiments (Figure 3). When the cells were pre-incubated for 24 h with a fixed concentration of antioxidants and then incubated with a toxic dose of oxidized LDL (200 μg apoB ml^{-1}), we observed a persistent protective effect of α-tocopherol and rutin. Since antioxidants and oxidized LDL were not in contact, these antioxidants could not be acting outside the cell by inactivating the toxic compounds contained in oxLDL. These data strongly suggest that α-tocopherol and rutin protect the cell directly by increasing the cellular resistance against the toxic effect of oxidized LDL.

Effect of α-tocopherol, ascorbic acid and rutin on peroxidation of cellular lipids and glutathione and ATP depletion

As shown in Figure 1, oxidized LDL induced an early rise of cellular TBARS and a late depletion of glutathione and ATP

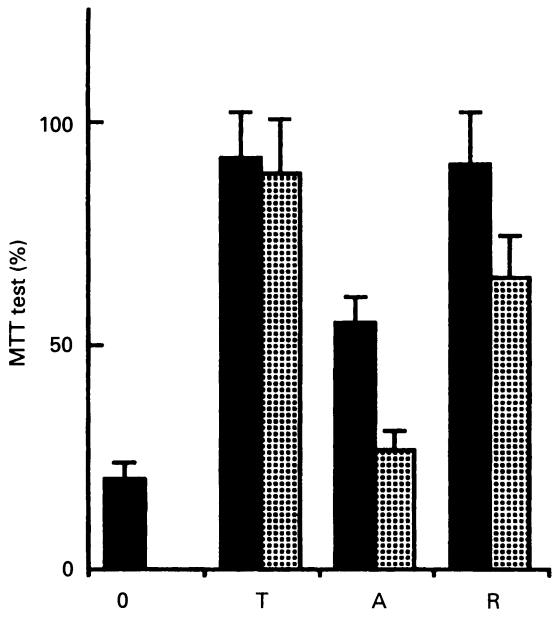

Figure 3 Persistence of the cytoprotective effect of antioxidants, 100 μmol l^{-1} α-tocopherol (T), 10 μmol l^{-1} ascorbic acid (A), 10 μmol l^{-1} rutin (R), in comparison to the control (O). Co-incubation experiments (antioxidants and oxidized LDL added simultaneously in the culture medium and incubated with cells for 48 h) were performed under the standard conditions (solid columns). In pre-incubation experiments (stippled columns), cells were incubated for 24 h in the presence of the same concentration of antioxidants, then the medium was replaced by a fresh standard medium (without any additional antioxidant) and cells were grown in the presence of oxidized LDL (200 μg apoB ml^{-1}) for an additional 48 h period. The cytotoxicity was determined by the MTT test (expressed as % of the control cells, grown in the absence of oxidized LDL or antioxidant) at the end of the 48 h incubation with oxidized LDL. Mean ± s.e.mean of 3 experiments (each point being done in duplicate).

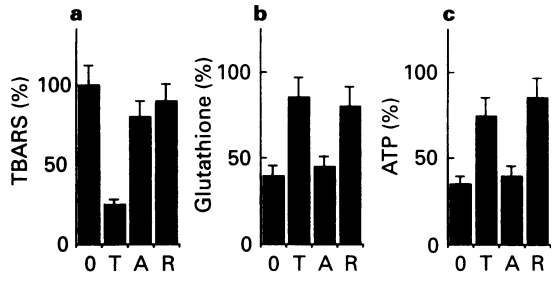

Figure 4 Effect of α-tocopherol, ascorbic acid and rutin on cellular TBARS formation (a), gluthatione (b) and ATP (c) depletion. Cells were incubated in the absence (O) or in the presence of antioxidants (T: 100 μmol l^{-1} α-tocopherol; A: 10 μmol l^{-1} ascorbic acid; R: 10 μmol l^{-1} rutin) and in the presence of oxidized LDL (200 μg apoB ml^{-1}) for 48 h. The cellular parameters were evaluated at the end of this period of incubation and expressed as a percentage of the control, i.e. cells grown under the standard conditions (in the absence of oxidized LDL and antioxidant). The results are expressed as percentage of controls (cells grown in the presence of native LDL). Mean ± s.e.mean of 3 separate experiments.

(these depletions being concomitant with the cytotoxicity). Protective concentrations of α-tocopherol inhibited the TBARS rise and the depletion of glutathione and ATP (Figure 4). In contrast, protective concentrations of rutin inhibited the glutathione and ATP depletion but not the TBARS formation (Figure 4). Ascorbic acid was relatively less effective on all the parameters. These data demonstrate that the rise in TBARS can be uncoupled from the cytotoxicity, thus suggesting that the peroxidation of cellular lipids is not the direct cause of cell death.

Uptake of oxidized LDL by endothelial cells in the presence of antioxidants

In order to exclude the possibility that the protective effect of α-tocopherol, ascorbic acid and rutin, could result from an inhibition of the uptake of oxidized LDL by endothelial cells, we determined the cellular uptake of oxidized LDL radiolabelled with the non-metabolizable [^{3}H]-oleyl cholesteryl ether. High (non toxic) concentrations of α-tocopherol (100 μmol l^{-1}), ascorbic acid (10 μmol l^{-1}) and rutin (10 μmol l^{-1}) did not inhibit significantly the uptake of mildly oxidized LDL (data not shown).

Discussion

We have previously shown that antioxidants could constitute two lines of defence against the injurious effect of LDL oxidation by inhibiting LDL oxidation or/and by increasing the cellular resistance against oxidized LDL. The mechanism of the latter effect is completely unknown. This led us to investigate the potential involvement of some possible cellular targets. In our experimental system, the relatively early rise of cellular TBARS may indicate a peroxidation of cellular lipids. Gluthathione and ATP depletion are relatively late events occurring at the time of the cytotoxicity. Our data on glutathione are consistent with those of Kuzuya *et al.* (1989) and Thomas *et al.* (1993) and may suggest the occurrence of a cellular oxidative stress during the cytotoxic process triggered by oxidized LDL. According to the general scheme of Boobis *et al.* (1989), depletion of cellular glutathione is associated with NADPH and ATP depletion, thiol protein oxidation and a defect of the cellular homeostasis. It was therefore of interest to try to block the cellular oxidative stress and to prevent its cellular consequences by use of antioxidants.

The direct protective effect of the tested antioxidants did not result from their interaction with LDL since, in preincubation experiments, they were in contact with cells but not with oxidized LDL. Similarly an inhibition of the uptake of oxidized LDL has been excluded. Although the precise intracellular mechanism(s) of the cytotoxicity of oxidized LDL is (are) unknown, the experiments reported here suggest that effective antioxidants act by blocking the intracellular events triggered by oxidized LDL. The inhibition of TBARS formation and of glutathione depletion by α-tocopherol is consistent with a block of the cellular oxidative process triggered by oxidized LDL. The mechanism of action of rutin is probably different since the depletion of glutathione and ATP was inhibited, but not the TBARS formation. Taken together, these data suggest that (i) the lipid peroxidation (TBARS rise) is not the direct cause of the lethal cellular damage; (ii) the late depletion of glutathione and ATP may be more directly involved in cell death, in agreement with the general scheme of the oxidative stress in cell injury (Boobis *et al.*, 1989).

Another important and novel conclusion is that α-tocopherol and rutin are able to prevent directly at the cellular level the cytotoxic effect of oxidized LDL by inhibiting the cellular oxidative stress generated by oxidized LDL. The biphasic protective/toxic effect of ascorbic acid in the presence of oxidized LDL was completely abolished when the 3 antioxidants were used in combination. These data are in agreement with the results obtained on biomembranes or liposomes (Leung *et al.*, 1981; Scarpa *et al.*, 1984; Wefers and Sies, 1988; Niki, 1989). All these results suggest the existence of an interaction between water-soluble (ascorbic acid and rutin) and lipid-soluble (α-tocopherol) antioxidants and that their combination could contribute to a better protection of the cell membranes.

In conclusion, we may emphasize that (i) protective doses of α-tocopherol and rutin are able to inhibit the glutathione and

ATP depletion triggered by toxic doses of oxidized LDL; (ii) antioxidant defences of endothelial cells can be effectively increased by pre-loading the cells with exogenous antioxidants. As antioxidants are able to increase *in vitro* the resistance of endothelial cells against the toxic effect of oxidized LDL, it may be speculated that they could also protect endothelium *in vivo* and thereby be anti-atherogenic, since the endothelial cell injury is thought to play a central role in atherogenesis (Ross, 1993). This protection of endothelium by antioxidants may explain at least in part the 'French paradox' (i.e. relatively low incidence of coronary artery disease in France compared with the other Western countries) which could be due to a relatively high dietary intake of antioxidants (from vegetables, fruits and red wines), (Frankel *et al.*, 1993). The present data suggest that a combination of antioxidants could be useful to protect endothelium and vascular walls against the oxidative injury.

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