If total (Ca^{2+}-dependent plus Ca^{2+}-independent) release was calculated as a percent of the radioactive GABA in the synaptosomes before perfusion, the values obtained on GABA derived from ABAL, from the medium and from glutamate were about 8.8, 13.2 and 22.5%, respectively.

A previous report by the authors [2] suggested that GABA in the GABA-T pool was degraded by GABA-T and released by a Ca^{2+}-independent mechanism, whereas GABA in the GAD pool was not in contact with GABA-T and was released by both a Ca^{2+}-independent and a Ca^{2+}-dependent mechanism. Furthermore, we argued that any movement of GABA between the pools was from the GABA-T pool to the GAD pool, with the movement unaffected by the size of the GABA-T pool. The results of the present study indicate that, like GABA captured from the medium, GABA derived from ABAL in the GABA-T pool is also subjected to active degradation by GABA-T, to release by a Ca^{2+}-independent mechanism, and to transport into the GAD pool.

The slightly lower release of GABA derived from ABAL compared with that of GABA taken up from the medium may be explained in the same way as its slightly lower degradation rate, that is, by presuming that ABAL is oxidized to GABA not only in synaptosomes that release GABA but also in synaptosomes that do not.

In summary, we report here that GABA formed from ABAL in synaptosomes was accumulated in a GABA-T pool and released in response to stimulation in the same way as GABA taken up from the medium, whereas GABA synthesized from glutamate went into a different pool. The physiological actions of GABA formed from ABAL in brain may be similar to those of GABA taken up from synaptic clefts.

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Protective effect of α-tocopherol, ascorbic acid and rutin against peroxidative stress induced by oxidized lipoproteins on lymphoid cell lines

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Lipid peroxides and oxygen reactive species are involved in major physiological or pathological events. The oxidative stress against unsaturated phospholipids can result in severe structural and functional damages in cell membranes [1] as demonstrated in the case of ischemia and vascular diseases [2–4].

Various antioxidants have been used for a long time in medical therapy of chronic venous insufficiency. The

rationale is to counteract the overproduction of oxygen reactive species which could be explained by the decrease in α-tocopherol content in varicose veins [4]. α-Tocopherol is considered to be an important lipid peroxide scavenger due to its solubility and occurrence in membrane [5]. Its level appears inversely related to lipoperoxidation processes in the organism or tissue submitted to oxygen stress. It could also stabilize biomembranes by forming complexes with the fatty acid moiety in the membrane lipid [6]. We recently reported a potentiation of anti-lipoperoxidative activities when α-tocopherol was combined with ascorbic acid and rutin in *in vitro* membrane-like systems [7].

A new experimental model system consisting of lymphoid cell lines cultured in the presence of UV-treated low density lipoproteins (LDL) has been recently designed to investigate the cytotoxic effects of lipid peroxides entering the cell through the endocytotic pathway [8, 9]. We have compared the protective activities of several antioxidants, used alone or in combination.

Materials and Methods

Lymphoid cell lines were established by Epstein–Barr virus (B95/8) transformation of blood B lymphocytes from healthy subjects as described previously [9]. Cells were grown in RPMI 1640 medium (Seromed, France) containing 10% fetal calf serum (Boehringer, France), penicillin (100 I.U./mL), streptomycin (100 μg/mL) and glutamine (0.3 mg/mL); 48 hr before LDL incorporation this medium was removed and replaced by RPMI 1640 containing 2% Ultroser HY (IBF, France) as a serum substitute.

Human plasma LDL ($1.006 < d < 1.063$) were prepared from pooled plasma of healthy subjects, by sequential ultracentrifugation (L70 Beckman Ultracentrifuge) in KBr, according to Havel *et al.* [10]. After dialysis against 150 mM NaCl containing 0.3 mM EDTA (pH 7.0), the LDL were oxidized by exposition to short UV-radiations (Osram lamp, 254 nm, 0.5 mW/cm^2) for 2 hr. LDL were then sterilized by filtration on a 0.2 μm Millipore membrane, and immediately incorporated into the cell culture medium. Their purity was assessed by polyacrylamide gel electrophoresis (Lipofilm Sebia, Paris).

The protective effect of α-tocopherol, *l*-ascorbic acid, *l*-ascorbic acid 6-palmitate, catechin, rutin and sodium rutin sulfate (all from the Sigma Chemical Co., St Louis, MO, U.S.A.) was determined by incorporating into the culture medium increasing concentrations of these compounds simultaneously with the oxidized LDL. Cells were suspended in fresh RPMI 1640 medium containing 2% Ultroser HY and tested antioxidants (0–500 μM), and distributed in Petri dishes (0.5–1 × 10^6 cells in 2 mL of culture medium per dish of 2.5 cm diameter). UV-treated LDL were added (150 μg apoB/mL) to the culture medium for a 48 hr incubation period.

The cell viability was determined, on an aliquot of the cell culture, by the trypan blue dye exclusion test. Cells were then pelleted by centrifugation (1000 rpm for 5 min), and 1.4 × 10^6 dpm/mL of [^{3}H]thymidine (25 Ci/mmol, CEA, France) was added to the culture medium 24 hr after the beginning of the pulse with oxidized LDL. Cells were grown for 24 hr in the presence of [^{3}H]thymidine, then were pelleted by centrifugation (1000 rpm for 5 min), washed twice in phosphate-buffered saline (pH 7.4) and homogenized by sonication (MSE sonicator) in 1 mL distilled water. An aliquot of the homogenate was used to determine, by liquid scintillation counting (Packard Tricarb 4530), the amount of [^{3}H]thymidine incorporated into the cell. The activity of LDH released into the supernatant was evaluated by a Roche kit assay (MA Kit 10).

Cell protection was also observed after a pre-incubation period with the antioxidant combination of α-tocopherol, ascorbic acid and rutin in a weight ratio 1:4:4. The antioxidant mixture was added to the culture medium for 48 hr, then the medium was removed and replaced by a medium containing oxidized LDL for a further 48 hr period of incubation.

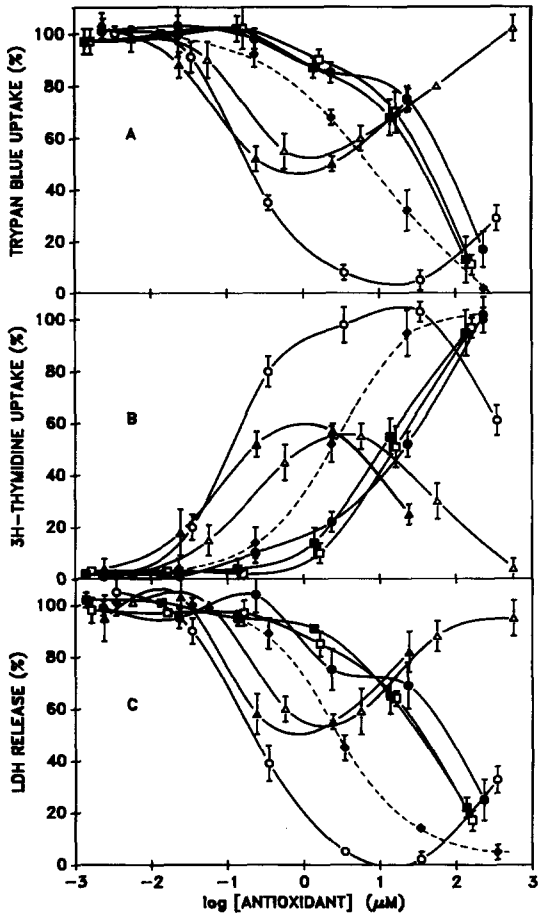

Fig. 1. Effects of antioxidants used alone (continuous lines), α-tocopherol (●), ascorbic acid (△), ascorbic acid palmitate (▲), catechin (○), rutin (□), sodium rutin sulfate (■), and the combination (dotted line) α-tocopherol/ascorbic acid/rutin (1:4:4) against peroxidative stress induced on lymphoid cell lines. Cells were exposed for 48 hr to oxidized LDL (150 μg apoB/mL) and increasing concentrations of antioxidants. The cytotoxicity was assessed by the trypan blue dye uptake (A), the thymidine incorporation (B) and the release of LDH (C) at the end of the incubation, and expressed as the percentage of control values obtained without antioxidant (100% is the maximal cytotoxicity of oxidized-LDL in the absence of any protective agent, and 0% is the ground level of cytotoxicity in the absence of oxidized LDL. Under the experimental conditions used, the respective trypan blue stained cells were 70 ± 15% and 12 ± 5%). Each value is the mean and SD from four experiments.

Results and Discussion

The cell protective effects of tested compounds against the peroxidative stress were reported in Fig. 1, and compared to values obtained without antioxidant addition. It should be noted that each viability parameter was consistent with each other.

A similar positive dose-dependent effect is noted for α-tocopherol, rutin and sodium rutin sulfate. A biphasic protective/toxic effect was observed for *l*-ascorbic acid, *l*-ascorbic acid 6-palmitate and catechin. To determine if this biphasic effect was due to a direct toxicity on lymphoid cells, antioxidants (0–500 μM) were added to the culture medium for 48 hr without oxidized LDL, then the cell viability was evaluated by the trypan blue uptake test: catechin was cytotoxic in this system for concentrations above 35 μM, while no toxicity was observed for ascorbic acid, α-tocopherol and rutin. These results are in agreement with those of Maridonneau-Parini *et al.* [11] who reported a damaging effect of catechin on erythrocyte membranes, whereas rutin was not toxic.

The protective effect of the triple combination α-tocopherol, ascorbic acid and rutin (concentration ratio 1:4:4) was also tested in this system. A positive dose–effect relationship is noted, and a higher cytoprotective activity is demonstrated by the leftward shift observed for the dose–effect curve, when compared to the dose–effect relationships noted for α-tocopherol and rutin alone (Fig. 1). Moreover, the biphasic protective/toxic effect previously noted for ascorbic acid disappeared when combined with α-tocopherol and rutin.

Besides, when the antioxidant mixture is added to the culture medium for 48 hr before the oxidative stress, the dose-dependent protective effect is similar (same dose–effect curve) to that recorded when antioxidants and oxidized LDL were added simultaneously.

The protective effect of α-tocopherol in our experimental system is quite consistent with that previously reported on synthetic or natural membranes [12]. α-Tocopherol has been shown to scavenge effectively oxygen reactive species [13] and lipid peroxyl radicals [5]. It was also suggested that it induces the formation within the architecture of the cellular membrane of a stereospecific slot leading to interactions of α-tocopherol with the arachidonyl chains of membrane phospholipids [14]. Such complexes were responsible for the stabilization of membranes exposed to the damaging action of free fatty acids [6]. Our results are also in agreement with the protection by vitamin E of cultured endothelial cells against the fatty acid hydroperoxide-induced injury reported by Hennig *et al.* [15].

Ascorbic acid and ascorbic acid palmitate exhibited in our cellular model system quite similar properties (protective effect at low concentrations and toxic effect at high concentrations, in the presence of oxidized LDL), in contrast with previous works reporting the more potent effect of ascorbic acid palmitate in a synthetic liposomal system [16] and in granulocytes [17]. The protective component of low amounts of ascorbic acid observed in our model could be related to the potent antioxidant properties of vitamin C [18]. The toxic effect of high concentrations of ascorbic acid observed in the presence of oxidized LDL did not result from a direct cytotoxicity of this antioxidant since the same concentrations of ascorbic acid were not cytotoxic in the absence of oxidized LDL. This suggests that the biphasic protective/toxic effect we observed could be explained by a biphasic redox property of ascorbic acid as previously demonstrated [12, 19].

When α-tocopherol, ascorbic acid and rutin are combined, they exhibit an obvious additional antilipoperoxidant effect and protect the cells against the peroxidative stress. This activity is higher than that observed when α-tocopherol and rutin are used alone, and the biphasic protective/toxic dose–effect is not evidenced. These data agree with results obtained on liposomes and biomembranes, and suggest that the balance between anti- and pro-oxidant effects of ascorbic acid can be shifted to the antioxidant side by sufficient amounts of vitamin E, as previously reported [12]. They are also consistent with the results demonstrating a mutual protective effect of α-tocopherol and ascorbic acid, and the suppression of their pro-oxidant activity [20], a cooperative interaction being well established in many different systems [13, 21].

A similar beneficial association between ascorbic acid and rutin has been demonstrated by Negre-Salvayre *et al.* [7] and Afanas'ev *et al.* [19]. Thus, these results suggest that an interaction occurs between water-soluble (ascorbic acid and rutin) and lipid-soluble (α-tocopherol) antioxidants, and that their combination contributes to a better protection of the cell. The last reported experiment (pre-incubation) demonstrates that:

1. The protective effect of the antioxidant mixture occurs inside or at the surface of the cell, with a true cytoprotective effect which cannot be explained only by an inhibition of the progression of LDL auto-oxidation, since in this experiment oxidized LDL were not in contact with the antioxidants incorporated into the culture medium.
2. The antioxidant defences of the cell can be effectively increased when cells are preloaded with exogenous antioxidants. This is quite consistent with the results showing a protection by α-tocopherol of cultured endothelial cells against lipid peroxide-induced injury [15].

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