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α-Tocopherol consumption during low-density-lipoprotein oxidation

Wendy JESSUP,*§ Sara M. RANKIN,†‖ Catherine V. DE WHALLEY,† J. Robin S. HOULT,† James SCOTT‡ and David S. LEAKE†¶

*Cell Biology Research Group, Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, †Pharmacology Group, Division of Biomedical Sciences, King's College, University of London, London, and ‡Division of Molecular Medicine, MRC Clinical Research Centre, Harrow, Middlesex, U.K.

1. The kinetics of the depletion of α-tocopherol in human low-density lipoprotein (LDL) were measured during macrophage-mediated and cell-free oxidation. The formation of oxidatively modified, high-uptake species of LDL in these systems was not detectable until all of the endogenous α-tocopherol had been consumed. 2. Supplementation of the α-tocopherol content of LDL by loading *in vivo* extended the duration of the lag period during which no detectable oxidative modification occurred. 3. The addition of a flavonoid (morin) prevented both α-tocopherol consumption and oxidative modification of LDL. 4. The α-tocopherol contents of LDLs from a range of individual donors could not be used to predict their relative resistance to oxidation, indicating that other endogenous antioxidants may also be present, and quantitatively significant, in human LDL.

INTRODUCTION

Low-density lipoprotein (LDL) is normally cleared from the circulation by endocytosis, mediated largely via cell-surface receptors which recognize binding domains on the apolipoprotein B-100 (apo B) component of the particle. Several types of chemical modification to apo B can reduce its affinity for the apo B/E receptor. Some, such as acetylation, can also promote binding of LDL to macrophage surface proteins (such as the 'scavenger' or acetyl-LDL receptor), leading to uncontrolled uptake and accumulation of large amounts of cholesterol by these cells [1]. Alterations to LDL during its incubation with certain cell types, such as endothelial cells, smooth muscle cells and macrophages, can also lead to accelerated LDL endocytosis and cholesterol accumulation by macrophages [2–5]. Evidence is accumulating that LDL oxidation occurs *in vivo* in atherosclerotic lesions [6], and this may therefore explain why cholesterol accumulates in foam cells derived from macrophages in these lesions.

Cell-mediated modification is believed to involve, and indeed be dependent upon, oxidation of LDL lipids [7]. Thus oxidation occurs in all cell-mediated incubations which lead to the formation of species of LDL which can be endocytosed rapidly by macrophage receptors, such as the 'scavenger' receptor. We will use the terms 'productive modification' and 'high-uptake LDL' to describe the process by which LDL is altered by cells and the species of LDL (taken up rapidly by macrophages) which is generated during this process respectively. If oxidation is suppressed by the addition to the culture medium of chain-breaking antioxidants (butylated hydroxytoluene, α-tocopherol or probucol) or metal chelators (EDTA or desferrioxamine), then productive modification is inhibited [3,7,8]. An apparently similar, but cell-free, productive modification can also be achieved by incubation of LDL with relatively high concentrations of redox-active metals such as copper or iron [3].

LDL contains a number of endogenous antioxidants, including vitamin E, β-carotene and retinyl esters [9]. It is likely that there are several more, as yet unidentified, lipophilic antioxidants present in the LDL core, perhaps derived mainly from dietary intake of natural and synthetic compounds. One might expect that the differences in the susceptibility of individual LDL preparations to oxidation, which have often been observed [9,10], are largely dictated by variations in the amounts of the various antioxidants that are present in these samples. It is also possible that differences in the incidence of heart disease between various national populations are at least partly dependent on differences in the dietary intake and associated lipoprotein content of lipid-soluble antioxidants such as vitamin E [11].

In this study we have examined the kinetics of α-tocopherol consumption during both cell-free (Cu^{2+}-stimulated) and cell-mediated oxidation of LDL. Simultaneous measurements were made of peroxidation and of the development of high-uptake properties of the LDL during incubations with macrophages. We were therefore able to compare the relationship between antioxidant consumption and oxidative modification of LDL. We also studied the relationships between natural and induced variations in the endogenous α-tocopherol content of LDL and the progress of LDL oxidation, and the influence of exogenous flavonoids, which are natural dietary components, on these events.

Abbreviations used: LDL, low-density lipoprotein; apo B, apolipoprotein B; PBS, Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

§ To whom correspondence should be addressed, at present address: Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, N.S.W. 2050, Australia

‖ Present address: Division of Endocrinology and Metabolism, Department of Medicine, University of California, San Diego, CA, U.S.A.

¶ Present address: Department of Biochemistry and Physiology, University of Reading, Reading, Berks., U.K.

MATERIALS AND METHODS

LDL preparation

LDL (density 1.019–1.050 or 1.063) was isolated from fresh human plasma collected in EDTA and aprotinin (final concentrations of 3 mM and 90 kallikrein inhibitory units/ml respectively) from normal healthy volunteers, by sequential density ultracentrifugation in KBr solutions at 10 °C. The LDL was washed by centrifugation at a density of 1.064, and then dialysed for 24 h against at least five changes of 50–100 vol. of phosphate-buffered saline (PBS; Dulbecco's A) containing EDTA (1.0 mg/ml) and chloramphenicol (0.1 mg/ml). All buffers were deoxygenated before use by bubbling with N_2, and the dialyses were performed under near-anoxic conditions in filled stoppered bottles at 4 °C.

Loading of LDL with α-tocopherol *in vivo* was achieved by oral dosage with commercially available D-α-tocopherol (free alcohol) at 1.45 g/day for 3 days. Samples of plasma were prepared from a single donor immediately before and after the treatment, and were used to prepare matched control and tocopherol-loaded LDL samples respectively.

Iodination of LDL was performed by the iodine monochloride method [12]. The iodinated LDL was separated from unreacted iodide on a column of Sephadex G-25 (PD-10; Pharmacia) and dialysed during 48 h against deoxygenated PBS containing EDTA (1 mg/ml) and chloramphenicol (0.1 mg/ml) at 4 °C.

Isolation of macrophages

Resident macrophages were isolated by peritoneal lavage of female Swiss TO mice with ice-cold PBS [13]. The cells were plated in 22 mm-diam. wells (Costar) in Dulbecco's modified Eagle's medium (DMEM; containing 1 g of glucose/l; Gibco) to which was added 10% (v/v) foetal calf serum (Gibco) and gentamicin (50 μg/ml), at 2×10^6 peritoneal cells/well for the cultures that were to modify LDL, and 1×10^6 cells/well for the macrophages that were to have modified LDL added to them. The cells were incubated for 2 h and then washed four times with PBS to remove contaminating non-adherent cells. Cultures were used immediately for modification of LDL, or after overnight incubation in DMEM containing 10% (v/v) foetal calf serum and 50 μg of gentamicin/ml for measurement of the uptake and degradation of modified LDL.

LDL oxidation

^{125}I-labelled LDL (100 μg of protein/ml; 0.6 ml/well) was incubated for up to 24 h in Ham's F-10 medium (Flow Laboratories), to which freshly prepared 3 μM-$FeSO_4$ had been added, either with modifying macrophages (macrophage-modified LDL) or in cell-free dishes (control LDL). The medium was then removed and centrifuged to remove any detached cells. In some cases, LDL (100 μg of protein/ml) was incubated in cell-free medium to which 100 μM-$CuSO_4$ had been added (copper-oxidized LDL). Controls were also prepared which lacked added redox-active metals. Samples of the LDL-containing media were diluted into fresh medium for measurement of uptake by cultured macrophages (see below). Other samples (pooled media from five replicate wells for each point) were mixed with butylated hydroxytoluene and EDTA (final concentrations of 20 μM and 2 mM respectively) and stored at 4 °C until the end of the experiment. These LDL samples were then either assayed directly for total hydroperoxide content, or extracted into heptane by the SDS method [14]. Heptane extracts were stored at −70 °C and assayed for lipid hydroperoxide and α-tocopherol content within 5 days.

Uptake of LDL by cultured macrophages

The various ^{125}I-LDLs were diluted to 10 μg of protein/ml with DMEM containing 10% (v/v) foetal calf serum and gentamicin (50 μg/ml), and incubated for 20 h with macrophages or in cell-free wells. Macrophages are unable to modify LDL in this medium [8]. The iodide-free trichloroacetic acid-soluble degradation products were measured [15]. Uptake was expressed as the amount of LDL degraded by macrophages, less the corresponding degradation products measured in the cell-free wells. The macrophages remaining in the wells were washed several times with PBS, dissolved in 0.2 M-NaOH and their protein content determined.

Autoxidation of LDL

LDL samples were diluted to 0.5 mg of protein/ml and subjected to auto-oxidation, essentially as described in [16]. Thus LDL solutions were placed in dialysis tubing (prepared by boiling for 30 min in distilled water) in a 4-fold volume of PBS containing 0.1 mg of chloramphenicol/ml. The buffer was bubbled continuously with water-saturated O_2 during the incubation. Previous studies have established that the slow oxidation of LDL which occurs under such conditions is dependent upon trace (i.e. micromolar) contaminating concentrations of redox-active metals present in the solutions [16].

Measurement of LDL peroxidation

The hydroperoxide contents of media were determined by a modification of the tri-iodide assay [17] adapted for use in an automated system [18]. Measurements were made of both aqueous samples and heptane extracts, to determine total and lipid hydroperoxides respectively. In general, the lipid hydroperoxides constituted in excess of 95% of the total hydroperoxides, and so only data for lipid hydroperoxides are provided here.

α-Tocopherol determination

Aliquots of heptane extracts were analysed by h.p.l.c. on a Merck Lichrocart CN column (250 mm × 4 mm) with hexane/propan-2-ol (99:1, v/v) as mobile phase and a fluoresence detector set to excitation at 295 nm and emission at 325 nm, essentially as described by Burton *et al.* [19], except that the column was run at room temperature and the flow rate was 1.5 ml/min. The system was calibrated on a daily basis using freshly prepared standard solutions of α-tocopherol in heptane. Preliminary studies established that the recovery of α-tocopherol during lipid extraction of LDL samples was essentially 100%.

Other determinations

Protein was determined according to Lowry *et al.* [20] or by a modification of this procedure [21].

RESULTS AND DISCUSSION

Kinetics of macrophage-mediated oxidation of LDL

When LDL is incubated with mouse peritoneal macrophages, it becomes modified so that it is 10–20 times more rapidly endocytosed and degraded by a second set of macrophages than control LDL incubated in medium alone; this property is retained when macrophage-modified LDL is re-isolated by centrifugation [8]. A typical example of the kinetics of formation of high-uptake LDL during incubation of labelled LDL with macrophages is shown in Fig. 1(*a*). As we have observed previously [8] there is a lag phase of 4–6 h before any detectable alteration occurs; thereafter, the development of high-uptake LDL proceeds at an approximately linear rate. No such 'productive' modification could be detected in the corresponding cell-free control incubations at any time up to 24 h (Fig. 1*b*).

The lag period during which no productive modification of LDL could be detected was a reproducible event in many separate experiments with cultured macrophages. It has also been reported to occur for endothelial-cell-mediated alterations to LDL [22], and it has been suggested that this lag period reflects the time during which endogenous antioxidants in LDL are oxidized. We therefore measured the time course of consumption of α-tocopherol in LDL during incubations with macrophages or cell-free wells, since this is the major endogenous antioxidant so far identified in LDL [16]. The mean α-tocopherol content of freshly isolated LDL from apparently normal donors was usually in the range 8–10 nmol/mg of LDL protein, which agrees well with other reports [16], and represents approx. 4–5 tocopherol molecules per LDL particle.

The α-tocopherol content of LDL declined rapidly during the early stages of incubation with macrophage cultures. Thus by 2–4 h, almost all of the α-tocopherol had disappeared from macrophage-exposed LDL, whereas in cell-free control incubations the consumption was much less rapid (Fig. 1). We assume that the accelerated depletion of α-tocopherol in cell-incubated LDL reflects the greater oxidative stress to which this LDL is exposed. No detectable transfer of this antioxidant from LDL to the cells occurs during the course of the incubation. The most striking feature of these experiments was the close relationship between the time at which α-tocopherol was exhausted and the appearance of high-uptake LDL in the medium bathing the cells. Fig. 1 shows the results of a single experiment in which the formation of high-uptake LDL followed rapidly after the depletion of α-tocopherol; this temporal relationship has been confirmed in several separate experiments. These results indicate that the endogenous antioxidant content of LDL may play an important role in the susceptibility of LDL to cell-mediated modification, although they do not indicate the proportional contribution of α-tocopherol to this process.

Hydroperoxides were detectable throughout the incubation periods from the earliest times examined. In the cell-mediated incubations, we assume that the hydroperoxides derive from oxidation of LDL lipids rather than from the cells themselves. In agreement with this, supernatants collected from macrophages incubated in the same medium, but in the absence of LDL, contained barely detectable amounts of hydroperoxides (results not shown). It appears that the endogenous lipid-soluble antioxidants are unable to suppress completely peroxidation induced by incubation of LDL in redox-active metal-containing media, in either the presence (Fig. 1*a*) or the absence (Fig. 1*b*) of modifying cells. We have also observed this phenomenon during 'autoxidation' of LDL [23], which is also dependent on

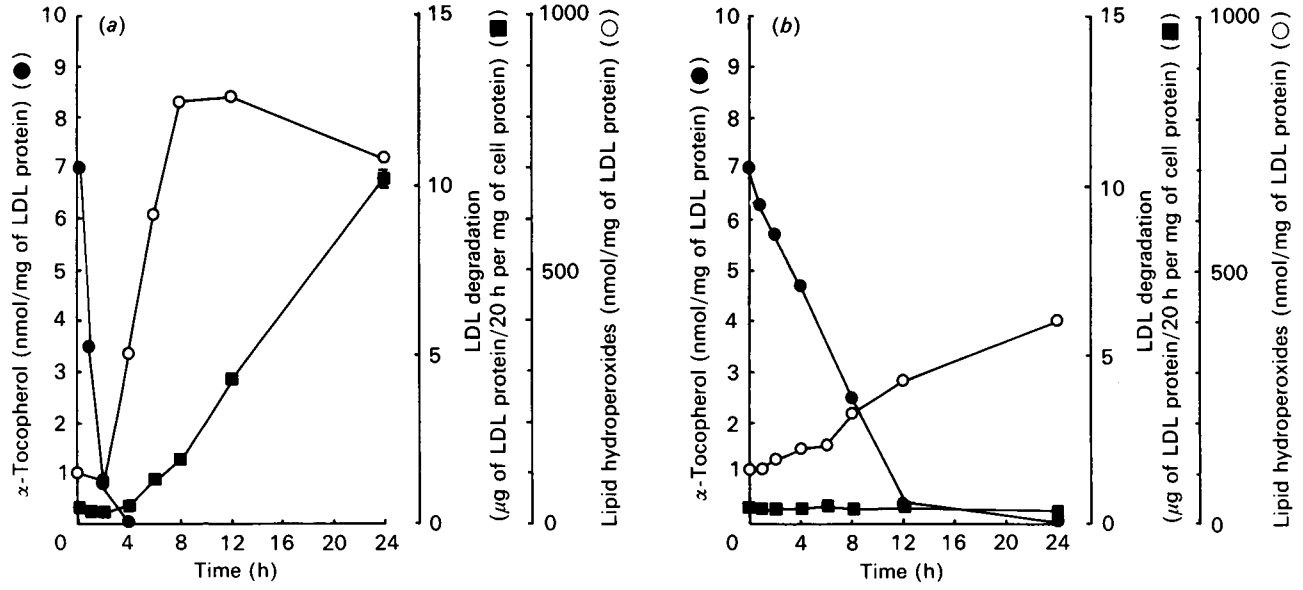

Fig. 1. Kinetics of macrophage-mediated oxidative modification of LDL

LDL was incubated (*a*) with macrophages or (*b*) in cell-free dishes in Ham's F-10 medium containing 3 μM-$FeSO_4$ for the times indicated, and then harvested. The α-tocopherol (●) and lipid hydroperoxide (○) content, and the rate of uptake and degradation by a second set of macrophages (■), were measured as described in the text. Data for degradation are means ± S.E.M. ($n = 3$); others are duplicate determinations (on pooled samples from five wells) which did not differ by more than 5%.

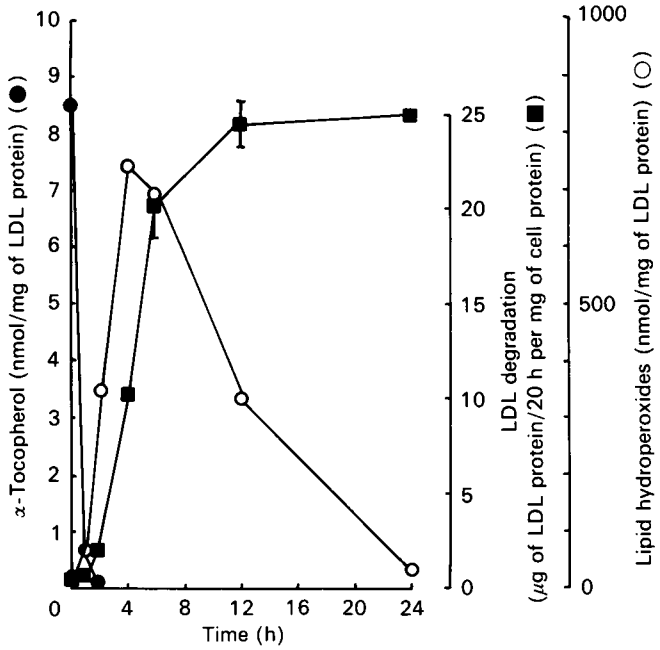

Fig. 2. Kinetics of copper-mediated oxidation of LDL

LDL was incubated in cell-free dishes containing Ham's F-10 medium plus 100 μM-$CuSO_4$ for the times indicated, then harvested and assayed for α-tocopherol (●), lipid hydroperoxides (○) and rate of degradation by macrophages (■). Data for degradation are means ± S.E.M. ($n = 3$); others are duplicate determinations (on pooled samples from five wells) which did not differ by more than 5%.

trace amounts of metal ions [16]. In contrast, during radiolysis of LDL in the absence of redox-active metals, very little oxidation of LDL can be detected until all of the α-tocopherol is consumed [24]. It is possible that the metal ions bind specifically to regions of the apo B molecule, catalysing the generation of a very local flux of radicals which are not so accessible to α-tocopherol as other regions of the LDL particle.

Incubation of LDL in a cell-free system in the presence of 5 μM-Cu^{2+} induced oxidative modification of LDL at approximately the same rate as the cell-mediated system described above [8]. When the Cu^{2+} concentration was increased to 100 μM (Fig. 2), the rate at which high-uptake LDL was generated was accelerated. A shorter lag phase was observed under these conditions, but this production of high-uptake LDL still closely matched the depletion of α-tocopherol in the LDL.

Modulation of endogenous α-tocopherol affects cell-mediated oxidation of LDL

If endogenous antioxidants are important in delaying the formation of high-uptake LDL, then it follows that LDL preparations containing elevated levels of antioxidants should be less susceptible to oxidative modification. This argument has previously been used to explain variations in the ease with which individual LDL preparations could be oxidized by cells [10], although we are not aware of any attempts to correlate such observations with direct measurements of antioxidant content. Direct addition of α-tocopherol to the medium of modifying cells inhibits oxidative modification [8], but it is not clear from these experiments whether the primary effect is on the LDL or the cells.

We have compared the progress of cell-mediated oxidation in normal and α-tocopherol-loaded LDLs. Samples of LDL were prepared from a single donor immediately before and after a period of oral dosage with D-α-tocopherol. This treatment led to a 2.5-fold increase in the α-tocopherol content of the loaded LDL compared with the matched control (22.0 nmol/mg of protein, cf. 8.62 nmol/mg of protein.) These LDL samples were labelled and incubated with macrophages or in cell-free wells (Fig. 3). In the early stages of the incubation, α-tocopherol was consumed at approximately the same rate in both control and loaded LDLs. Because of its higher initial α-tocopherol content, the complete depletion of α-tocopherol in the loaded LDL occurred 2 h later than in the control LDL. We also found a similar 2 h retardation in the peroxidation of the samples, and a matching increase in the lag period before the onset of accelerated uptake of the LDL by macrophages.

Thus an increase in the α-tocopherol content of LDL (under circumstances where other variables are probably largely unaffected, from a single donor dosed with α-tocopherol) increases the resistance of LDL to cell-mediated oxidation. This is consistent with epidemiological data [25] which show an inverse correlation between plasma vitamin E levels and the incidence of coronary deaths in several European populations. An increase in the resistance of LDL to oxidation may mean that less LDL may be modified locally in the arterial wall during its normal residence time there.

Effects of exogenous flavonoids on cell-mediated oxidation of LDL

Flavonoids are a large family of benzo-γ-pyrone derivatives of widespread occurrence in plants. Considerable amounts are consumed in the normal diet [26]. Several flavonoids are known to be effective peroxyl radical scavengers [27,28]. They are also reported to influence cyclo-oxygenase, lipoxygenase, phospholipase A_2 and NADPH oxidase activities [29–31]. We have shown that low concentrations of flavonoids are potent inhibitors of cell-mediated formation of modified high-uptake LDL [32,33]. Fig. 4 shows the results of a typical experiment in which we measured the effect of the addition of a flavonoid (1 μM-morin) to the culture medium on the kinetics of LDL oxidation. The addition of morin to the system completely suppressed the development of high-uptake forms of LDL by macrophages (Fig. 4*c*). This was associated with complete suppression of hydroperoxide formation (Fig. 4*a*) and with maintenance of the α-tocopherol content of the LDL (Fig. 4*b*).

Flavonoids also effectively inhibited cell-free oxidation of LDL by 100 μM-Cu^{2+} [33], suggesting that their ability to inhibit macrophage-mediated LDL oxidation is possibly due largely to their radical-scavenging properties. Also, macrophage-mediated modification is not influenced by cyclo-oxygenase inhibitors [33], suggesting that morin does not act by inhibiting this enzyme, a conclusion supported by a recent study showing that morin is only a weak cyclo-oxygenase inhibitor [30]. Flavone, which has the flavonoid nucleus but does not

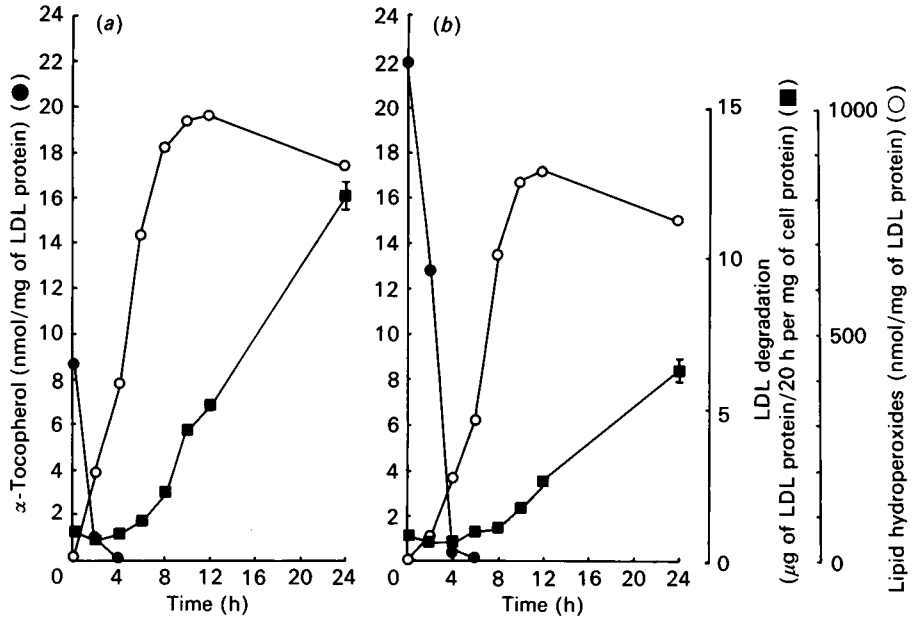

Fig. 3. Effects of dietary α-tocopherol supplementation on the kinetics of macrophage-mediated LDL oxidation

LDL was isolated from a single donor both immediately before (*a*) and after (*b*) a 3-day period of oral supplementation with α-tocopherol (1.45 g/day). The LDLs were iodinated, incubated with macrophages or with cell-free dishes (results not shown) for the times indicated, then harvested and assayed for α-tocopherol (●) and lipid hydroperoxide (○) content and for rates of degradation by a second set of macrophages (■). Data are means ± S.E.M. ($n = 3$) for degradation; others are means of duplicate determinations (on pooled samples from five wells) which did not differ by more than 5%. No high-uptake LDL was detected in any of the cell-free dishes (results not shown).

contain any phenolic groups and is not a good radical scavenger, but which is a potent inhibitor of cyclo-oxygenase [30], did not inhibit macrophage-mediated oxidation [33]. The present study therefore confirms previous observations [7,34] that exogenously supplied lipophilic antioxidants can also protect LDL against oxidative damage, and raises the interesting possibility that flavonoids may be naturally occurring anti-atherosclerotic agents in the diet if they reach sufficiently high levels in the plasma.

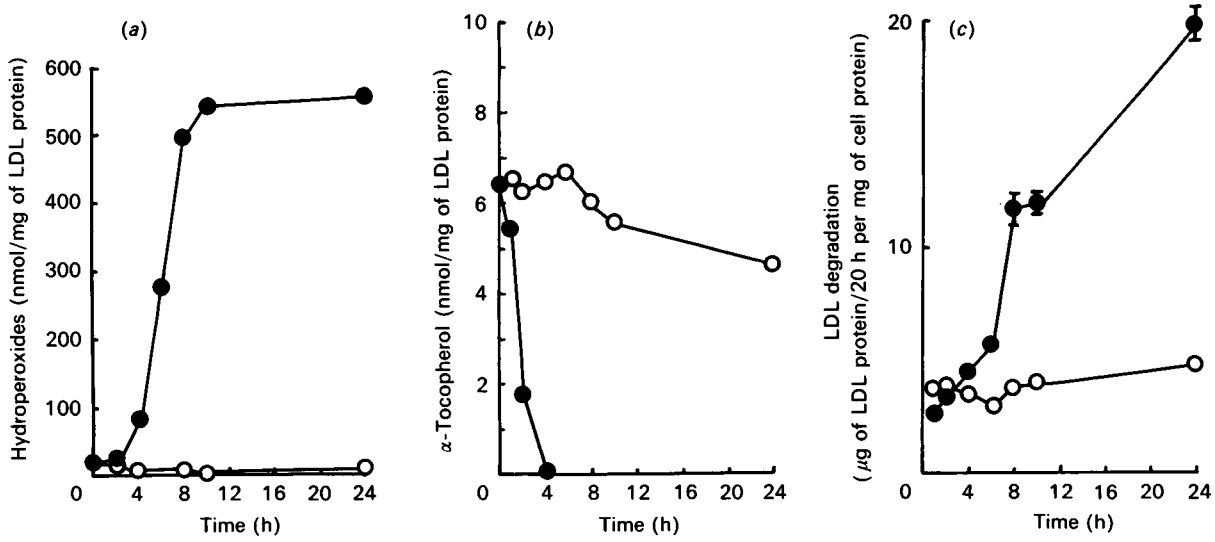

Fig. 4. Effect of a flavonoid (morin) on the progress of macrophage-mediated LDL oxidation

LDL was incubated with macrophages (*a*,*b*,*c*) or cell-free dishes (results not shown) in the presence (○) or absence (●) of 1 μM-morin for the periods shown. Morin was prepared as a 1 mM stock solution in ethanol and diluted into the medium. The final concentration of solvent was 0.1% (v/v). Control incubations contained ethanol only, at the same concentration. The media were harvested and assayed for (*a*) lipid hydroperoxide, (*b*) α-tocopherol, and (*c*) rate of degradation by a second set of macrophages. Data are means ± S.E.M. for degradation ($n = 3$); others are means of duplicate determinations (on pooled samples from five wells) which did not differ by more than 7%.

Table 1. α-Tocopherol status and susceptibility of LDL to oxidation in a range of donors

For macrophage-mediated oxidation expts., LDL samples were iodinated and subjected to macrophage-mediated oxidation and its assay as described in the Materials and methods section. For auto-oxidation expts., LDL samples were diluted to 0.5 mg of protein/ml and incubated for 24 h under conditions which promote mild autoxidation, as described in the Materials and methods section. Data are from a single experiment and are the means of duplicate determinations which did not differ by more than 8%. n.m., not measured; n.d., not detectable; LOOH, lipid hydroperoxides.

		Macrophage-mediated oxidation		Auto-oxidation	
Donor	α-Tocopherol (nmol/mg of LDL protein)	LOOH (nmol/mg of LDL protein)	Lag period (h)	LOOH (nmol/mg of protein)	α-Tocopherol remaining at 24 h (nmol/mg of protein)
A	3.53	524	3	127	n.d.
B	5.70	n.m.	n.m.	108	2.99
C	4.97	n.m.	n.m.	120	3.64
D	14.10	693	3	290	0.67
E	8.89	n.m.	n.m.	32	7.20

The α-tocopherol content of LDL is not predictive of its resistance to oxidative damage

Whereas endogenous α-tocopherol almost certainly performs a significant function in the protection of LDL against oxidative stress *in vitro*, and may also be important in prevention of atherogenesis *in vivo*, we anticipate that other endogenous antioxidants are also important contributors to the resistance of LDL to oxidation. Thus when LDLs prepared from a range of donors, including some with high (14.1 nmol/mg of LDL protein) and some with low (3.53 nmol/mg of LDL protein) α-tocopherol levels were subjected to macrophage-mediated or cell-free Cu^{2+}-mediated oxidation, no correlation could be found between these levels and the rate of peroxidation in a cell-free system (Table 1). We performed a detailed kinetic study (results not shown) of the macrophage-mediated oxidation of samples A and D (see Table 1) but could detect no differences in the duration of the lag period before productive modification could be detected, or in the subsequent rates of peroxidation and productive modification. From this we deduce that other endogenous antioxidants are likely to be present in LDL and to contribute to its defence against oxidative stress and its consequences. Some of these have already been identified [9], and it is possible that there are others. The levels of individual lipid-soluble antioxidants are probably dictated largely by diet and probably vary independently between donors. This may explain why measurement of the α-tocopherol content of LDL preparations may not describe their total antioxidant content, nor predict their resistance to oxidative stress, even though elevation of the α-tocopherol content of a single source of LDL (in which the dietary intake of other antioxidants remains approximately constant) clearly affects the kinetics of its oxidation and modification *in vitro* (Fig. 3). An additional source of variation may be in the content of peroxidizable lipids in different LDL preparations, which will also be greatly affected by diet and likely to vary between individuals. In future it may be more useful to determine the content of a range of endogenous antioxidant compounds in studies such as this, and a more general assay of the LDL antioxidant status, such as the TRAP (total peroxyl radical trapping antioxidant parameter) assay [35] may be more useful for determination of the overall antioxidant content and of sensitivity to oxidation of individual LDL preparations. It may also be necessary to monitor the precise lipid composition of LDLs rather more closely, particularly with respect to their unsaturated lipid content.

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