α-Tocopherol supplementation of macrophages does not influence their ability to oxidize LDL

Anna Baoutina,[1] Roger T. Dean, and Wendy Jessup

Cell Biology Unit, The Heart Research Institute, 145 Missenden Road, Camperdown, New South Wales 2050, Australia

Abstract We have investigated the effect of α-tocopherol-loading of mouse peritoneal macrophages and human monocytes on their ability to oxidize human low density lipoprotein (LDL). Mouse peritoneal macrophages incorporated α-tocopherol (α-TOH) from culture medium supplemented with the vitamin in a time- and concentration-dependent manner. Subcellular fractionation by density gradient ultracentrifugation showed that the distribution of incorporated α-TOH within the cell was similar to that of free cholesterol. Most (≈88%) of α-TOH partitioned into the membrane fractions (plasma membrane ≈41%, mitochondria and lysosomes ≈26%, and endosomes plus endoplasmic reticulum ≈21%). Cellular α-TOH was stable for at least 24 h in serum- or LDL-free media whether permissive (Ham's F-10) or non-permissive (Dulbecco's minimum essential medium, DMEM) for LDL oxidation. When incubated with LDL in DMEM, α-TOH-preloaded cells transferred small amounts of α-TOH (approximately 1 nmol/mg LDL protein after 9 h) to the lipoprotein. However, enrichment of the cells with α-TOH did not change the kinetics of oxidation of either normal or TOH-depleted LDL in Ham's F-10 medium compared with non-loaded cells, as assessed by α-TOH consumption, cholesteryl ester degradation, and cholesteryl ester hydroperoxide and 7-ketocholesterol accumulation. Nor did it alter superoxide release by the cells or their ability to reduce extracellular copper(II). Similar to mouse macrophages, enrichment of human monocytes with α-TOH did not change the kinetics of cell-mediated LDL oxidation. We conclude that elevated cellular levels of α-TOH in mouse peritoneal macrophages and in human monocytes do not affect their ability to oxidize LDL lipids in vitro. This suggests that either cell-mediated oxidation of LDL under the conditions of this study is not dependent on cell-derived radical species or that cellular α-TOH is unable to affect their formation.—**Baoutina, A., R. T. Dean, and W. Jessup.** α-Tocopherol supplementation of macrophages does not influence their ability to oxidize LDL. *J. Lipid Res.* 1998. **39:** 114–130.

Supplementary key words atherosclerosis • LDL oxidation • cells • macrophages • monocytes • α-tocopherol • antioxidants

Low density lipoprotein (LDL) is the major carrier of cholesterol in human blood plasma. There is a growing body of evidence that oxidation of LDL is likely to play a key role in the early stages of atherogenesis (1, 2). In vitro studies have shown that oxidation of LDL leads to formation of modified lipoprotein particles with pro-atherogenic properties (1, 3). These include cytotoxicity, stimulation of monocyte recruitment and their adhesion to endothelial cells, and the rapid uptake of oxidatively modified LDL by macrophages and smooth muscle cells resulting in formation of lipid-laden foam cells. Several lines of evidence support the idea that oxidative modification of LDL may also occur in vivo. Oxidized lipids and proteins, as well as oxidatively modified LDL, have been found in atherosclerotic lesions from both experimental animals and humans (1, 4–6). Furthermore, lipophilic antioxidants have been shown to retard the progression of dietary-induced atherosclerosis in some animal models (1, 7, 8). Some recent epidemiological studies suggest an association between increased intake of natural plasma antioxidants, especially vitamin E, and decreased risk of cardiovascular disease (1, 9, 10).

Due to the abundance of antioxidant defenses present in blood plasma, it is unlikely that significant

Abbreviations: LDL, low density lipoprotein; α-TOH, α-tocopherol; HBSS, Hank's balanced salt solution; HEPES, N-[hydroxyethyl]piperazine N′-[ethanesulfonic acid]; PBS, phosphate-buffered saline; GSH, glutathione; BSA, bovine serum albumin; BCS, bathocuproine disulphonic acid; SOD, superoxide dismutase, PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum; DMEM, Dulbecco's minimum essential medium; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; AA, arachidonic acid; AAPH, 2,2′azo*bis*(2-amidinopropane)hydrochloride; MPM, mouse peritoneal macrophages; hMON, human monocytes, FC, free cholesterol; CE, cholesteryl ester; CE 18:2, cholesteryl linoleate; CE 20:4, cholesteryl arachidonate; CEOOH, cholesteryl ester hydroperoxide; 7-KC, 7-ketocholesterol; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; LOX, lipoxygenase; TBARS, thiobarbituric acid reactive substances; CL, chemiluminescence; HPLC, high performance liquid chromatography.

[1]To whom correspondence should be addressed.

LDL oxidation occurs in the circulation. It is believed that oxidative modification of LDL is more likely to occur within the intima of the arterial wall (1, 6). It has been shown that the cells present in the arterial intima and developing lesions (macrophages, monocytes, endothelial cells, smooth muscle cells, and lymphocytes) possess the capacity of oxidizing LDL in vitro (1, 6), although the precise mechanism(s) by which these cells promote LDL oxidation remains elusive (11). Numerous studies have shown that various lipid- and water-soluble antioxidants (vitamin E, butylated hydroxytoluene (BHT), probucol, ethylenediaminetetraacetic acid (EDTA), ascorbate, desferrioxamine, etc.) attenuate cell-mediated LDL oxidation when added to the extracellular medium (12, 13). Under these conditions it is not clear whether such antioxidants exert their effect within the lipoprotein particle, in the medium, or at the cellular level. In some studies the endogenous antioxidant content of LDL was manipulated by in vivo or in vitro supplementation (or depletion) of various antioxidants (14–19). Although in vivo loading of LDL by oral supplementation with α-TOH increased the resistance of lipoprotein to macrophage- or endothelial cell-mediated oxidation, no correlation has been found between the level of α-TOH in LDL in non-supplemented humans and its sensitivity to cell-mediated oxidation (14, 16). Moreover, under mild oxidizing conditions, α-TOH may also act as a pro-oxidant increasing LDL oxidizability (20–22), and dietary supplementation or in vitro enrichment of LDL with vitamin E can, in some cases, result in more rapid initiation of oxidation of LDL in the presence or absence of cells (18, 19).

Despite a great body of work on the ability of lipid-soluble antioxidants to influence lipoprotein oxidation both in vivo and in vitro, little attention has focused on how the antioxidant content of cells could affect their ability to promote LDL oxidation, and the degree to which manipulation of the cellular antioxidant level could influence their pro-oxidant capacity. Evidence has been obtained that part of the inhibitory effect of probucol on cell-mediated LDL-oxidation may be exerted within the modifying cells (23). It has been recently reported that loading J774 macrophages with pharmacologically high concentrations of vitamin E can protect LDL against macrophage-mediated oxidation (17). In the present study, we manipulated the α-TOH content of mouse peritoneal macrophages and human monocytes by incubating them in the medium supplemented with α-TOH within a more physiologically relevant range and studied the stability of α-TOH in cells under different conditions. We also examined the possibility of transport of α-TOH between the cells and LDL particles, and investigated whether the cellular level of α-TOH affects their ability to promote LDL oxidation.

MATERIALS AND METHODS

Chemicals and reagents

Hank's balanced salt solution (HBSS, phenol red-free) and phosphate-buffered saline (PBS) (both Ca^{2+}- and Mg^{2+}-free), glutathione (GSH), bovine serum albumin (BSA) (Fraction V), d-α-tocopherol, L-ascorbic acid, bathocuproine disulphonic acid (BCS), bovine erythrocyte copper/zinc containing superoxide dismutase (SOD), phorbol 12-myristate 13-acetate (PMA) and cytochrome *c* (from bovine heart) were purchased from Sigma. Ham's F-10 medium, RPMI 1640 and fetal calf serum (FCS) were obtained from GibcoBRL. Dulbecco's minimum essential medium (DMEM) and N-[hydroxyethyl]piperazine N′-[ethanesulfonic acids] (HEPES) were from Trace BioSciences and ICN, respectively. EDTA, BHT, and potassium bromide were from BDH; chloramphenicol was from Boehringer Mannheim. 2,2′-Azo*bis*(2-amidinopropane)hydrochloride (AAPH) was from Polysciences; Ebselen (2-phenyl-1,2-benzoisoselenazol) and arachidonic acid (AA) were purchased from Cayman. Nanopure water was used for preparation of all aqueous buffers, PBS was subsequently treated with washed Chelex-100 resin (Bio-Rad) to remove contaminating amounts of transition metals (24). PD-10 columns prepacked with Sephadex G-25 (Medium) were obtained from Pharmacia Biotech. HPLC-grade hexane, methanol, and isopropanol were from Mallinckrodt or EM Science; ethanol (analytical grade) was from BDH. All other chemicals used were of the highest purity available. White-cell concentrates (<24 h ex vivo) and human serum (used in human monocyte isolation) were kindly provided by the N.S.W. Red Cross Blood Transfusion Service, Sydney, Australia.

Cell culture

Resident macrophages (MPM) were isolated from six-week-old Quackenbush Swiss strain mice, after CO_2 asphyxiation, by peritoneal lavage with ice-cold DMEM, containing 0.38% (w/v) sodium citrate, penicillin G (100 units/ml), and streptomycin (100 μg/ml). The isolated cells were immediately plated in 12-well (22-mm diameter) cell culture plates (Falcon) at 4×10^6 cells per well or, for subcellular fractionation experiments, in 100-mm diameter tissue culture dishes at 20×10^6 cells per dish. The cells were incubated at 37° for 2 h and then washed three times with PBS to remove

non-adherent cells. Monocytes were isolated from white-cell concentrates by using countercurrent centrifugal elutriation essentially as described previously (25). In brief, white-cell concentrates were diluted 1:2 in elutriation medium (HBSS containing 100 mg/l EDTA and 1% human serum). Peripheral-blood mononuclear cells (PBMC) were isolated after underlaying 30 ml of diluted white-cell concentrate with 15 ml of Lymphoprep (Nycomed, Oslo, Norway) and centrifuging at 400 *g* for 40 min at 21°C. The harvested PBMC (final volume 50 ml) were loaded into the elutriation chamber in a Beckman JE-6 rotor in a Beckman J2-21 M/E centrifuge at a speed of 2020 rev/min with an increasing flow rate (25). The purified monocyte fraction was >95% esterase-positive (Sigma, catalog no. 181B). After washing in RPMI 1640, the monocytes were plated in 12-well cell-culture plates at 1×10^6 cells per well, adhered for 2 h, and then washed with PBS to remove non-adherent cells. The cells were cultured in a humidified incubator at 37°C in 5% CO_2 in air. All tissue culture media were supplemented with 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cell viability was assessed by the trypan blue exclusion test.

Loading cells with α-TOH

An appropriate amount of α-TOH in ethanol was added to heat-inactivated FCS; the solution was briefly stirred and incubated by shaking at 37°C for 15 min in the dark. DMEM (or RPMI for hMON) was added, mixed vigorously, and the mixture was further incubated at 37°C for 15 min with shaking. The resulting medium was sterilized by passing through a 0.45-μm filter. The concentration of FCS in the final medium was 10% (v/v) and the amount of ethanol did not exceed 0.1% (v/v). Control medium contained 10% FCS in DMEM plus the appropriate amount of ethanol.

One ml of α-TOH-enriched medium was added to adhered and washed (as above) MPM or hMON. After various incubation times, the medium was removed and the cells were washed twice with PBS (37°C) containing 0.25% (w/v) BSA, followed by two washes with PBS alone. This procedure removed α-TOH non-specifically bound to the cell surface (26) and to the tissue culture dishes (data not shown). Control (non-loaded) cells were incubated for the same period in control medium.

In the studies of the stability of α-TOH in α-TOH-enriched cells or of the transfer of α-TOH between cells and LDL particles, MPM were further incubated with 1.5 ml of a serum-free medium (Ham's F-10 or DMEM) or DMEM containing LDL (100 μg protein/ml) for indicated times.

Subcellular fractionation

After incubation with α-TOH-enriched medium (120 μM α-TOH) for 18 h, MPM from 10 dishes (20×10^6 cells per dish) were washed as described above and then washed twice with ice-cold HBSS. The cells were removed by scraping and centrifuged at 145 *g* for 15 min. The cells were then resuspended in homogenization buffer (0.25 M sucrose, 20 mM HEPES, 0.5 mM EDTA, pH 7.0, 4°C) and ruptured using a shear force device (27). The homogenate was centrifuged at 800 *g* and 4°C for 5 min to remove unbroken cells, cellular debris, and nuclei. The supernatant was fractionated using a 1–22% Ficoll gradient underlaid with a 45% Nycodenz cushion by centrifugation in a Beckman VTi 65.2 rotor at 240,000 *g* and 4°C for 90 min (27). Twenty-six 200-μl fractions were collected from the bottom of the tube. A 150-μl portion of each fraction was extracted with methanol–hexane for lipid analysis as described below. Each fraction was also assessed for protein and for aryl sulfatase (27) and alkaline phosphatase activity (28) as marker enzymes for lysosomes and plasma membranes, respectively.

Preparation of LDL

Blood was collected from fasted normolipidemic healthy volunteers in 50-ml tubes, containing EDTA, aprotinin (90 kallikrein inhibitory units/ml, Sigma) and protease inhibitors, soybean trypsin inhibitor (20 μg/ml, Calbiochem) and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, 100 μM, Calbiochem). Plasma was prepared by centrifuging blood at 1,600 *g* for 20 min at 10°C. LDL (d 1.02–1.05) was isolated by density gradient ultracentrifugation (Beckman L8-M ultracentrifuge) using a vertical (VTi50) rotor for 2.5 h at 242,000 *g* (mean) and 10°C (24). A second centrifugation at a density of 1.063 g/ml with Ti70 rotor (242,000 *g* (mean), 22 h, 10°C) removed traces of contaminating albumin. The LDL was subsequently dialyzed for 18 h at 4°C against 4×1 liter of deoxygenated PBS containing chloramphenicol (0.1 g/l) and EDTA (1.0 g/l), filter-sterilized (0.45-μm) and stored in the dark at 4°C until use (within 7 days). Some of the LDL was used to prepare α-TOH-depleted LDL. Immediately prior to oxidation or incubation with cells, LDL was further dialyzed at 4°C against 4×1 liter PBS containing chloramphenicol (0.1 g/l) and Chelex-100 (4 g/l) to remove EDTA (24).

In vitro preparation of α-TOH-depleted LDL

α-TOH-depleted LDL was prepared as described in detail in (19). LDL (≈2–4 mg/ml) was passed through two consecutive PD-10 columns equilibrated with PBS and oxidized with 50 mM AAPH at 37°C. At various

times, aliquots of LDL were removed and placed on ice and AAPH was removed from the samples by two sequential gel filtrations using the PD-10 columns. The small amounts of lipid hydroperoxides accumulated in lipoproteins during the AAPH oxidation step were reduced to the corresponding non-reactive hydroxides by the treatment of the LDL with GSH and Ebselen, as described previously (30). Excess of Ebselen and GSH was removed by passing lipoproteins through two PD-10 columns. Control LDL samples were treated in the same way except that they were incubated with an equal volume of PBS instead of AAPH for the same periods of time. The time for complete α-TOH consumption varied between 20 to 30 min for different LDL preparations. This procedure allowed in vitro preparation of peroxide-free, α-TOH-depleted LDL without significant changes in the lipid composition (19).

Oxidation of LDL

Aliquots (1.5 ml) of Ham's F-10 medium containing 150 μg LDL were incubated at 37°C in 22-mm tissue culture wells (Falcon) in 5% CO_2 in air, with or without cells. Three separate wells were used for each condition.

Lipid extraction

At the times indicated, the LDL-containing media were removed from the cell culture wells and spun in an Eppendorf centrifuge at 16,000 *g* for 2 min at 4°C to remove any detached cells. One-half ml of the supernatant (or cell-free medium) was mixed with 50 μl of 0.2 mM BHT and 50 μl of 20 mM EDTA. One ml of cold methanol was added and the tube was shaken for 10 s. Hexane (5 ml) was added next and the tube was shaken again (30 s) and centrifuged (1,000 *g*, 5 min, 10°C). Four ml of the hexane phase was withdrawn, evaporated under vacuum, and redissolved in 200 μl of isopropanol. Samples were sealed in glass vials and stored at -80°C until HPLC analysis (within 7 days). Recovery of α-TOH and lipid in the extracts was ⩾99%; α-TOH, cholesteryl esters (CE) and their hydroperoxides (CEOOH) were stable under this condition for at least 6 weeks (data not shown).

The cells were washed three times with warm PBS, and the cell lipids were extracted twice with 1 ml of hexane–isopropanol 3:2 (v/v). Recovery of lipid of interest in the extracts was 98–100%, as judged by the extraction of a lipid film made by evaporation of a mixture of the standard lipid solutions in the culture well. Pooled extracts were evaporated under vacuum and redissolved in 200 μl of isopropanol. The cells were then lyzed by incubation in 0.5 ml 0.2 M NaOH for 15 min at 4°C and the lysates were stored at -20°C for protein assay (within 7 days).

HPLC analyses

Lipid extracts from cells or LDL-containing media were analyzed using reverse-phase HPLC with a Supelco ODS column (25 cm × 0.46 cm, 5 μM particle size) with a 2-cm Pelliguard guard column as described (31) using a mobile phase of ethanol–methanol–isopropanol 19.5:6:1 (v/v/v) containing 5 mM lithium perchlorate. Analysis was performed using electrochemical (for α-TOH, detection limit ≈0.1 pmol) and UV_{210} (for free cholesterol (FC) and CE) detection. CEOOH were analyzed by post-column chemiluminescence (CL) detection (detection limit ≈1–5 pmol) (31). In some experiments, HPLC analysis was performed as described previously (32). In brief, the lipids were separated on ODS column using acetonitrile–isopropanol 30:70 (v/v) as a mobile phase with UV_{210} detection for unoxidized FC and CE. Analysis of their oxidation products, 7-ketocholesterol (7-KC) and CEOOH, was performed separately using acetonitrile–isopropanol–water (144:27:1 (v/v/v) and UV_{234} detection. Quantitation of FC, individual CE, and 7-KC was performed using calibration curves for each of the commercially available compounds. CEOOH were quantified using a cholesteryl linoleate hydroperoxide standard prepared as described previously (33).

Measurement of superoxide production by macrophages

Superoxide radical production was measured as the SOD-inhibitable reduction of cytochrome *c* (34). Control or α-TOH-enriched MPM (4×10^6 cells/well) were washed three times with PBS (37°C), then incubated with 1 ml HBSS containing 80 μM cytochrome *c* in the presence or absence of SOD (10 μg/ml) at 37°C for 90 min. Some incubations were performed in the presence of PMA (4 μg/ml). Cell-free controls were run in parallel. The culture supernatants were placed on ice, then centrifuged to remove any detached cells and cytochrome *c* reduction was measured at 550 nm. Three separate wells were used for each condition in two independent experiments.

Measurement of copper reduction by macrophages

MPM (4×10^6 cells/well) were washed three times with PBS (37°C) and subsequently incubated at 37°C in 1 ml HBSS containing 50 μM $CuCl_2$ and 125 μM BCS for 90 min (35). This had no effect on cell viability. Parallel control incubations were performed in the absence of cells. The culture supernatants were removed, centrifuged to remove any cells, and their absorbance was measured at 482 nm. The concentration of copper reduced by the cells was calculated using an extinction coefficient $\epsilon = 12.2\ mM^{-1}\ cm^{-1}$ for the BCS-Cu(I)-

complex (36). The total recovery of copper after the incubation of its solutions with or without cells was estimated after the reduction of all copper in the supernatants to Cu(I) with an excess of ascorbate (100 μM); this was between 94 and 100%. Three separate wells were used for each condition in two independent experiments.

Cellular lipoxygenase activity assessment

The activity of 12/15-lipoxygenase (LOX) in MPM was estimated by quantitative analysis of 12- and 15-hydroxyeicosatetraenoic acids (12- and 15-HETEs), the products of AA which formation is catalyzed by the enzyme. Control or α-TOH-enriched macrophages, cultured and washed as above, were incubated in 1 ml of DMEM with or without AA (100 μM; in ethanol) for 1 h at 37°C. Cell-free incubations were run in parallel. The supernatants were collected and spun in centrifuge filter units (0.22 μM, Costar) to remove cellular debris. Direct analyses of 12- and 15-HETEs and the corresponding HPETEs without prior extraction was performed by a reverse-phase HPLC method, previously described (37). In brief, 100 μl of the supernatants was loaded onto an ODS column (as above) and eluted with a mobile phase of acetonitrile–methanol–water–acetic acid 35:15:25:0.1 at 1.5 ml/min. Analysis was performed using UV_{234} detection. The cells were washed and extracted with hexane–isopropanol as described above, followed by NaOH-lysis for protein assay. The extracts were evaporated, redissolved in 200 μl of the mobile phase, and analyzed for HETEs and HPETEs. 12- and 15-H(P)ETEs were quantified using corresponding standards obtained from Cayman. Every condition was run in duplicate.

Protein assay

The protein content of LDL samples and cell lysates was measured using the bicinchoninic acid method (Sigma) with BSA as a standard. BSA standards were prepared in water or in 0.2 M NaOH for the LDL preparations or cell extracts, respectively. The samples were incubated for 60 min at 60°, and the absorbance at 562 nm was measured.

Precipitation of LDL

Precipitation of LDL from conditioned medium was performed by the dextran sulfate–$MgCl_2$ method (38). Equal volumes of dextran sulfate (20 g/L) and $MgCl_2$ (1M) were combined and 100 μl of the mixture was added to 1 ml of LDL-containing medium (100 μg LDL protein). After standing at room temperature for 10 min the samples were centrifuged at 1,500 *g* for 30 min at 4°C. The supernatant was carefully removed, the lipids were extracted with methanol–hexane (as above) and analyzed as detailed above.

RESULTS

Loading mouse peritoneal macrophages and human monocytes with α-TOH: cellular distribution of α-TOH in loaded MPM

The α-TOH content of freshly isolated and adhered MPM was between 23 and 75 pmol/mg cell protein (mean 35.8 ± 20.6 pmol/mg, n = 6) and the ratio of intracellular α-TOH to FC in the range of 1:2326 to 1:714 (mol/mol) (mean 1:1052, n = 6).

Incubation of the cells with α-TOH-enriched me-

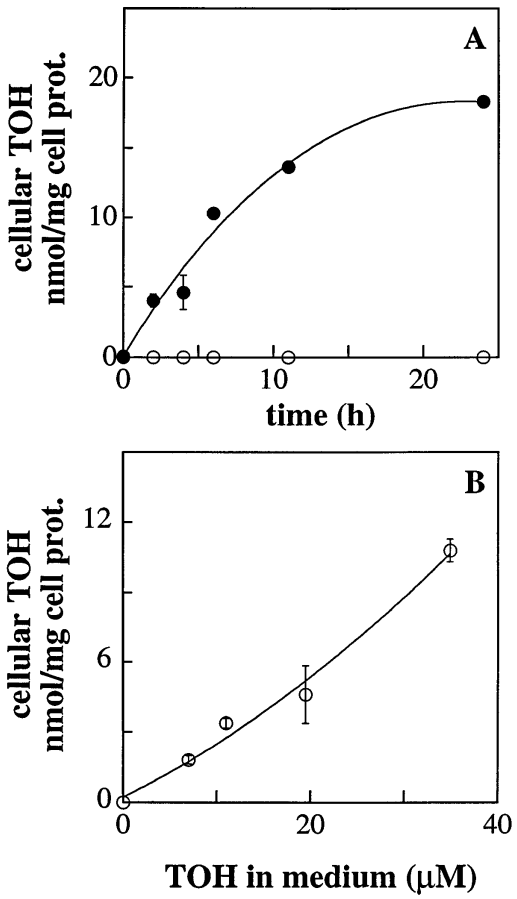

Fig. 1. Incorporation of α-TOH into MPM. A: Macrophages were incubated with medium containing 20 μM of α-TOH (●) or control medium (○) for the times indicated (see Materials and Methods). The concentration of ethanol in all media was 0.1% (v/v). B: Macrophages were incubated with loading medium, containing 0, 7, 11, 19.5 or 35 μM of α-TOH for 4 h. Values are mean ± SD for triplicate cultures.

dium (measured concentration of α-TOH in the medium 20 μM) resulted in a time-dependent incorporation of α-TOH into the cells (**Fig. 1A**). Cellular α-TOH increased proportionally with incubation time for about 8 h, with a decline from linearity after that time. Cellular FC and recovered protein were not altered by the loading of MPM with α-TOH (data not shown). Similar time-dependent incorporation of α-TOH into MPM was observed with the loading medium containing 40 μM α-TOH, although higher levels of cellular α-TOH were achieved (data not shown). Incubation of MPM with medium containing various concentrations of α-TOH for 4 h revealed a dose-dependent uptake of α-TOH by the cells (Fig. 1B). α-TOH was not toxic to the cells within the range of the concentrations and incubation times used in this study (up to 350 μM in the culture medium), as assessed by a dye exclusion test and maintenance of normal cell protein in the culture. Ethanol in the concentrations not exceeding 0.1% (v/v) of total medium also had no detectable effect on these parameters (data not shown). The ratio of total cellular α-TOH to FC achieved after 16 h loading of MPM with α-TOH depended on the level of α-TOH in the medium, and varied between 1:3 to 1:1.3 (mol/mol) for concentrations of α-TOH in the range of 20 to 40 μM. In the following experiments, MPM were loaded with α-TOH by their incubation in the medium containing 20 μM α-TOH for 16 h unless specified otherwise.

After α-TOH loading, the subcellular distributions of α-TOH and cholesterol were measured. Cells were homogenized and fractionated on 1–22% sucrose density gradient as described in Materials and Methods. We have previously characterized distribution of MPM organelles in this system using enzyme markers, iodination of cell surface proteins, and distribution of protein throughout the density gradient (27). The cellular distribution of FC was not altered by loading the cells with α-TOH (data not shown). The distribution of the incorporated α-TOH within α-TOH-loaded MPM was similar to that of free cholesterol (**Table 1**). Most of α-TOH (≈88%) associated with the membrane-containing organelles with three major pools being in the plasma membrane (41% of cellular α-TOH), mitochondria and lysosomes (26%), and endosomes plus endoplasmic reticulum (21%). This indicates that α-TOH incorporated into cells was not restricted to the cell surface, but distributed throughout the cell membranes in a manner similar to that of cholesterol.

TABLE 1. Subcellular distribution of α-tocopherol in α-TOH-loaded MPM

Fraction	TOH Distribution	Cholesterol Distribution
	% of total	
Cytosol	11.3 ± 3.0	7.6 ± 3.0
Plasma membrane	41.1 ± 3.4	49.9 ± 6.5
Lysosomes plus mitochondria	26.1 ± 6.1	19.2 ± 5.5
Endosomes plus endoplasmic reticulum	21.1 ± 0.9	21.2 ± 1.7

MPM were loaded with α-TOH by incubating the cells for 20 h in the medium containing 40 μM α-TOH. The cells were washed and subjected to the density gradient subcellular fractionation as described in Materials and Methods. Amounts of α-TOH and FC in each fraction were measured (in nmol/fraction) and expressed as % of total cell homogenate loaded on the gradient (total values for α-TOH and FC per cell protein were 58.6 ± 24.1 nmol/mg and 60.3 ± 19.3 nmol/mg, respectively). Values are means ± SD of three independent experiments.

The α-TOH content of freshly isolated human monocytes was higher than in MPM and varied between 0.3 and 0.4 nmol/mg cell protein (mean 0.34 ± 0.045 nmol/mg, n = 4); the ratio of intracellular α-TOH to FC being in the range of 1:127 to 1:338 (mol/mol) (mean 1:279, n = 4). The level of α-TOH in hMON increased to 2.96 ± 0.35 nmol per mg cell protein (n = 6) after 18 h incubation of the cells in the medium containing 23 μM α-TOH and to 19.1 ± 0.2 (n = 3) in the medium with 200 μM α-TOH. The resultant ratio of cellular α-TOH to FC for these loading conditions was, correspondingly, 1:37 and 1:5.16.

Stability of α-TOH in cultured cells: possible exchange of α-TOH between the cells and LDL particles

The stability of α-TOH after its incorporation into macrophages was studied. MPM enriched with α-TOH were further incubated in 1.5 ml serum-free "permissive" (Ham's F-10) or "non-permissive" (DMEM) medium. The terms "permissive" and "non-permissive" medium relate to the ability of the medium to support cell-mediated LDL oxidation (11); a key feature of permissive media is their content of redox-active transition metals (11, 39, 40) (for Ham's F-10 this is 3 μM of Fe(III) and 0.01 μM of Cu(II). In both media, the cellular content of α-TOH in the enriched MPM remained stable for at least 24 h (data not shown).

To investigate the possible effect of the cells loaded with α-TOH on the α-TOH content of LDL, both control and α-TOH-loaded macrophages were incubated with serum-free DMEM in the presence of LDL. In the case of control cells, there was a small decrease in α-TOH content of LDL within the first 2 h (**Fig. 2A**) without any changes in the levels of FC or CE in LDL (data not shown). This was not accompanied by the accumulation of any detectable α-tocopheryl quinone (α-TQ), one of the possible products of α-TOH oxidation, in the medium. At the same time, a slight increase of α-TOH level in the cells (Fig. 2B) was observed. These results together with the observation of unchanged levels of FC and CE in the medium during the incubation of

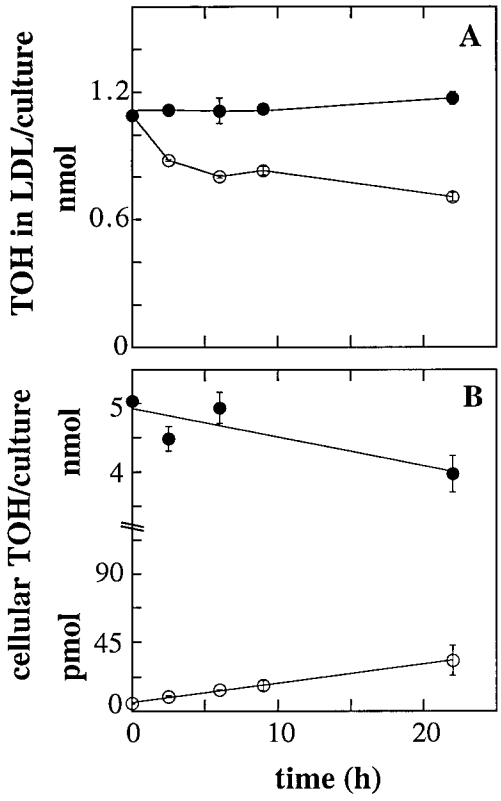

Fig. 2. α-TOH content of LDL and MPM during their incubation in DMEM. LDL (100 μg/ml) was incubated in DMEM with control (○) MPM or MPM enriched with α-TOH (40 μM α-TOH in the medium, 16 h) (●). After the incubation times indicated the medium was withdrawn, the cells were rinsed, and both the medium (A) and the cells (B) were extracted for lipid analysis. Values are mean ± SD for triplicate cultures.

cells with LDL might suggest a possible direct transfer of α-TOH from LDL to the cells, rather than cellular uptake of LDL as a whole. This may be a consequence of the different starting levels of α-TOH in LDL and MPM (approx. 1 nmol versus 5 pmol per culture, respectively). However, this transfer alone is not sufficient to quantitatively account for the observed decrease in α-TOH in LDL, as the latter (≈300 pmol per culture by 22 h of incubation) was 10-fold greater than the quantity of α-TOH accumulated in MPM over the same period (≈28 pmol per culture). The possible additional processes involved in decrease of α-TOH in LDL under current conditions were not further investigated in this study. Among them could be reactions involving traces of transition metals in LDL or in the medium, and/or traces of pre-formed lipid hydroperoxides in LDL (mean 2.55 ± 1.97 nmol/mg LDL protein, n = 6); these reactions might be facilitated by cells.

In contrast, when LDL was added to cells preloaded with α-TOH, the concentration of α-TOH in LDL was almost unchanged for at least 22 h (Fig. 2A). At the same time the level of α-TOH in the cells decreased slightly by 22 h (Fig. 2B), which again was not accompanied by the formation of any detectable α-TQ. These data indicate a net loss of α-TOH from macrophage cultures containing LDL. The loss occurs predominantly from the quantitatively major source of α-TOH in the system, whether this is LDL (in the experiments with control cells) or cells (with α-TOH-enriched cells). Given the stability of α-TOH in α-TOH-loaded cells in LDL-free medium, it is possible that the maintenance of α-TOH in LDL in the presence of α-TOH-enriched cells was due to direct transfer of α-TOH from cells to LDL.

To further investigate whether α-TOH-loaded macrophages can donate α-TOH to LDL, we performed similar experiments using LDL that was depleted of its TOH. The initial concentration of endogenous α-TOH in in vitro TOH-depleted LDL was below the detection limit (≈10 pmol α-TOH per mg of LDL protein). Incubation of α-TOH-enriched MPM with TOH-depleted LDL in DMEM resulted in a virtually linear increase in α-TOH concentration in the medium, which reached ≈1 nmol/mg LDL protein by 9 h incubation (data not shown). When expressed in nmol of α-TOH per culture, the amount of α-TOH acquired by TOH-depleted LDL was similar to that lost by the α-TOH-enriched MPM during their incubation, i.e., between 2 and 9 h of incubation, α-TOH level in the LDL increased by 0.064 ± 0.012 nmol, in comparison with 0.056 ± 0.1 nmol α-TOH lost by the cells.

To investigate whether the α-TOH transferred from the cells to the medium was physically associated with LDL, α-TOH-enriched MPM were incubated with α-TOH-depleted LDL for 22 h. The medium was then collected and α-TOH concentrations in the medium before and after LDL precipitation were compared. The results of this experiment indicated that more than 90% of α-TOH in the medium co-precipitates with LDL using the dextran sulphate–$MgCl_2$ method (data not shown).

Effect of enrichment of MPM and hMON with α-TOH on their ability to promote LDL oxidation

It has previously been shown that mouse peritoneal macrophages accelerate LDL oxidation in Ham's F-10 medium (14). It is believed that the presence of trace amounts of transition metals in the medium is an absolute requirement for the promotion of LDL oxidation by non-stimulated cells (11, 39, 40). To investigate whether the α-TOH level of the cells influences the rate of LDL oxidation, we compared the kinetics of LDL oxidation in the presence of cells with different α-TOH contents. Control and α-TOH-enriched macro-

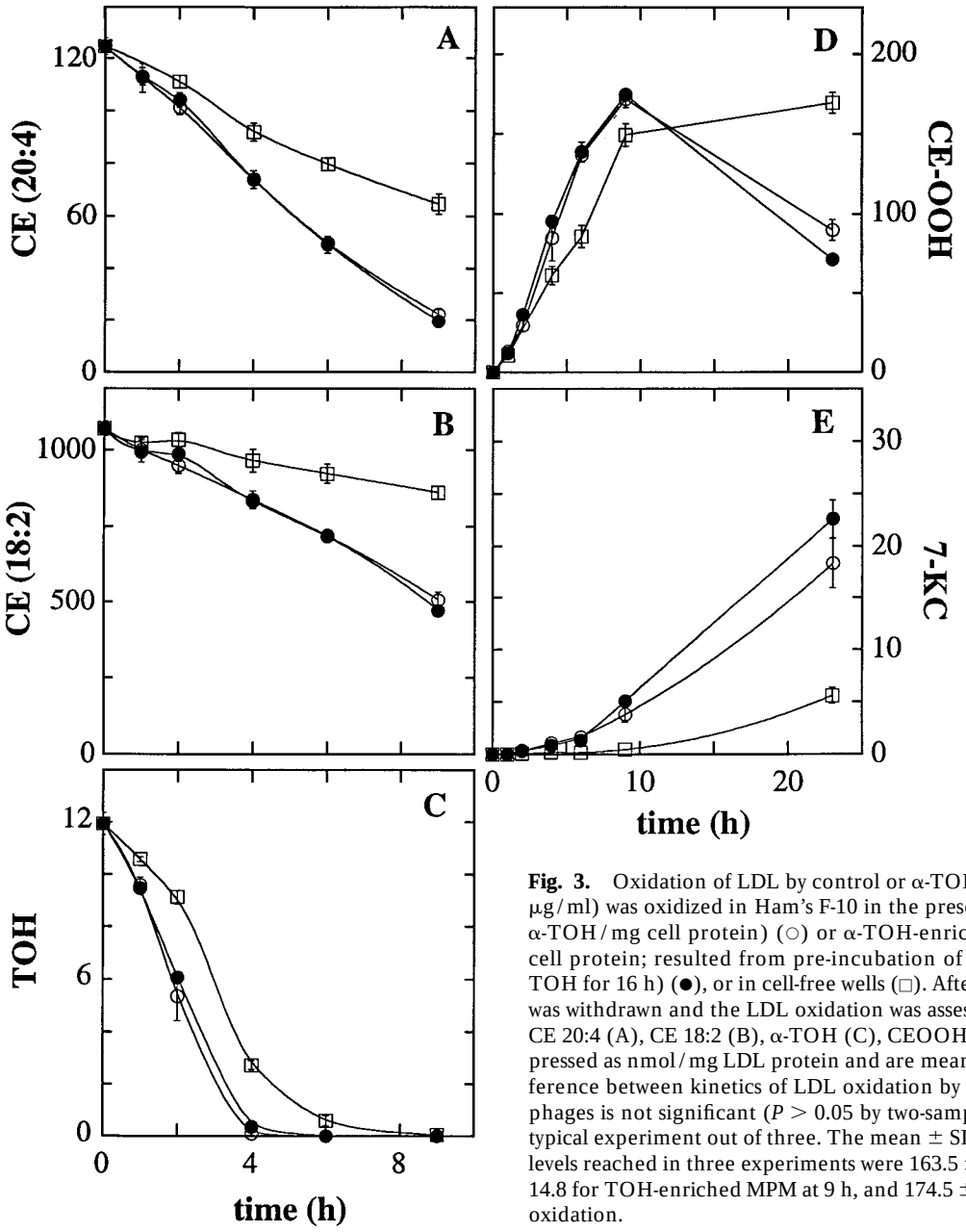

Fig. 3. Oxidation of LDL by control or α-TOH-loaded macrophages. LDL (100 μg/ml) was oxidized in Ham's F-10 in the presence of control MPM (27 pmol of α-TOH/mg cell protein) (○) or α-TOH-enriched MPM (31 nmol α-TOH/mg cell protein; resulted from pre-incubation of MPM in medium with 30 μM α-TOH for 16 h) (●), or in cell-free wells (□). After the times indicated the medium was withdrawn and the LDL oxidation was assessed by changes in the contents of CE 20:4 (A), CE 18:2 (B), α-TOH (C), CEOOH (D), and 7-KC (E). Values are expressed as nmol/mg LDL protein and are mean ± SD for triplicate wells. The difference between kinetics of LDL oxidation by control vs. α-TOH-loaded macrophages is not significant ($P > 0.05$ by two-sample *t*-test). The data are from one typical experiment out of three. The mean ± SD of maximum values for CEOOH levels reached in three experiments were 163.5 ± 7.9 for control cells and 157.2 ± 14.8 for TOH-enriched MPM at 9 h, and 174.5 ± 5.3 for cell-free condition at 24 h oxidation.

phages were incubated with LDL in Ham's F-10 medium; cell-free control incubations were included in each experiment (**Fig. 3**). LDL oxidation was assessed by measurement of several parameters: the loss of cholesteryl linoleate (CE 18:2) and cholesteryl arachidonate (CE 20:4), as two quantitatively major substrates for LDL lipid oxidation, consumption of LDL α-TOH (the major lipid-soluble antioxidant in LDL), and the accumulation of lipid oxidation products. These were CEOOH, primarily derived from CE 18:2 and CE 20:4, and 7-KC. We have previously demonstrated that accumulation of CEOOH at early stages of cell-mediated LDL oxidation is followed by their decomposition with formation of secondary lipid oxidation products in LDL (14, 32). Thus, determination of CEOOH with a very sensitive CL detection was used to assess LDL oxidation at its early stages, while at more advanced stages (when CEOOH levels decline), the accumulation of the oxysterol, 7-KC, becomes an important marker of oxidative modification of LDL (32).

Figure 3 shows that LDL oxidation was more rapid in the presence of MPM than in their absence, as indicated by increased rates of α-TOH consumption, CE degradation, and the generation of both CEOOH and

7-KC. When LDL was oxidized in the presence of α-TOH-enriched cells, changes in the concentrations of α-TOH, CE, CEOOH, and 7-KC in LDL occurred at rates very similar to those in LDL exposed to non-loaded cells (Fig. 3). Further, cells containing higher amounts of α-TOH (65 nmol per mg cell protein; achieved by pre-incubation of MPM in the loading medium containing 70 μM α-TOH) oxidized LDL at the similar rate as control cells and as the cells containing 31 nmol α-TOH/mg cell protein (data not shown). Similar results were obtained in three separate experiments using different LDL and MPM preparations. Within each experiment the kinetics of LDL oxidation by α-TOH-supplemented cells did not differ from control cells by more than 3.6%. It should be noted that although the kinetics of LDL oxidation varied between the individual experiments (the time of onset of oxidation and the rate of propagation) as we have previously established (14), the maximum accumulation of CEOOH was normally reached between 6 to 9 h and the coefficient of variation between three separate experiments did not exceed 9.4%. Increase of α-TOH in the loading medium to supraphysiological levels (260 μM or 350 μM) led to accumulation of correspondingly higher amounts of α-TOH in the cells (96 and 118 nmol α-TOH/mg cellular protein and ratios of α-TOH to FC of 3.3 and 3.9, respectively). However, oxidation of LDL by these cells, as assessed by loss of cholesteryl esters and accumulation of corresponding hydroperoxides, also occurred at a rate similar to that of control cells (data not shown). This was despite a slower rate of α-TOH loss in LDL in the presence of macrophages enriched with α-TOH at these high concentrations (data not shown). The latter might have reflected contributions of two independent processes which affect the level of α-TOH in LDL: the consumption of α-TOH during LDL oxidation in Ham's F-10 and transfer of α-TOH from α-TOH-enriched cells to LDL, as suggested earlier. An additional result in support of this transfer was an increase of α-TOH content of LDL incubated in Ham's F-10 with macrophages containing high levels of α-TOH at advanced stages of oxidation after it had been almost completely oxidized at earlier time points. Thus, after 6 h of oxidation by MPM with 118 nmol α-TOH/mg cell protein, the LDL α-TOH dropped from the starting concentration of 11.5 ± 0.4 nmol/mg LDL to 0.6 ± 0.1 nmol/mg LDL; by 22 h of incubation its concentration increased to 3.0 ± 0.2 nmol/mg LDL.

Similar results were obtained with TOH-depleted LDL. Mouse peritoneal macrophages actively oxidized TOH-depleted LDL, as indicated by more rapid loss of CE and accumulation of CEOOH than in the cell-free conditions (**Fig. 4**). However, loading of macrophages with α-TOH did not result in any significant changes in their ability to oxidize such LDL (Fig. 4): the variability between the kinetics of LDL oxidation by control and α-TOH-enriched cells did not exceed more than 8.6% for any of the parameters studied and statistically the differences were not significant (the lowest *P* value being 0.129, i.e., >0.05, by two-sample *t*-test). This was again despite the apparent transfer of small amounts of α-TOH to LDL particles from the α-TOH-enriched cells, as indicated by a slight increase in α-TOH concentration in TOH-depleted LDL after its incubation with α-TOH-loaded cells for 24 h (Fig. 4D). It should be noted that kinetics and degree of oxidation of separate preparations of TOH-depleted LDL varied significantly in three individual experiments, e.g., the maximum in CEOOH formation mediated by cells was reached at 4 h in one experiment, and at 7 and 24 h in two others. This variability can be explained by the different profile of modifications in lipids in LDL particles achieved during oxidation of normal LDL from different donors with AAPH in the process of preparation of TOH-depleted LDL on different occasions (see Materials and Methods).

To test whether the lack of the effect of cellular α-TOH level on kinetics of cell-mediated LDL oxidation was specific for mouse peritoneal macrophages, we performed similar experiments with human monocytes. It has previously been demonstrated that freshly isolated hMON promote oxidation of LDL in Ham's F-10 medium (41; B. Garner, personal communication). Indeed, oxidation of LDL, as measured by consumption of its α-TOH, loss of CE 20:4 and CE 18:2, and formation of CEOOH and 7-KC occurred at a higher rate in the presence of 1×10^6 hMON than in cell-free conditions (**Fig. 5**). Similar to MPM, control and α-TOH-enriched monocytes (10-fold difference in intracellular content of α-TOH) oxidized LDL at the same rate (Fig. 5). The difference between kinetics of LDL oxidation by control and α-TOH-loaded monocytes in the three independent experiments did not exceed 6.2%. Furthermore, the kinetic of LDL oxidation by monocytes containing 19.1 nmol of α-TOH per mg of cell protein (62 times higher than in control cells, resulted from incubation of hMON in the medium containing 200 μM α-TOH) was almost identical to that in the presence of non-loaded cells (data not shown). As was pointed out above for MPM, kinetics of LDL oxidation by human monocytes varied between the individual experiments with different LDL and cell preparations: the degree of variability of mean values for CEOOH for three experiments with human monocytes was between 9 to 21% for different time points, and within 10.5% for cholesteryl esters.

These results indicate that elevated α-TOH levels in

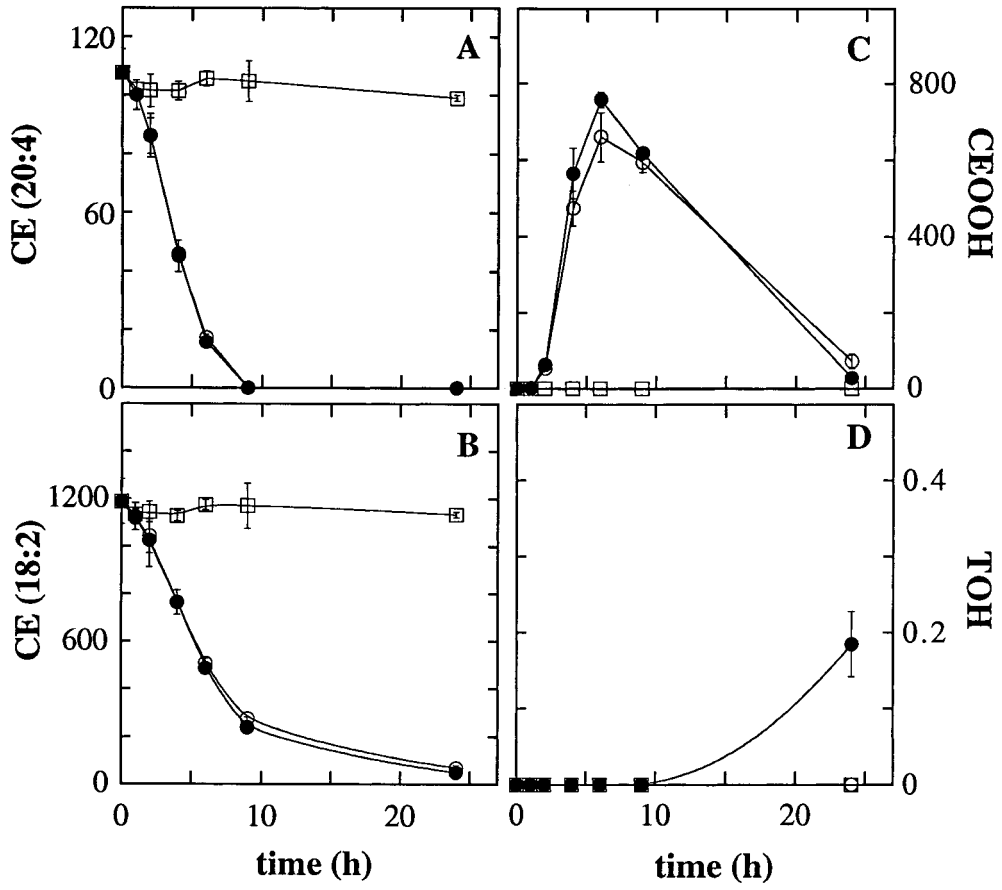

Fig. 4. Oxidation of TOH-depleted LDL by control or α-TOH-loaded macrophages. TOH-depleted LDL (100 μg/ml) was oxidized in Ham's F-10 in the presence of control (non-detectable level of α-TOH) (○) or α-TOH-enriched MPM (23 nmol α-TOH/mg cell protein) (●), or in cell-free wells (□). After the times indicated the medium was withdrawn and the LDL oxidation was assessed by changes in the content of CE 20:4 (A), CE 18:2 (B), CEOOH (C) and α-TOH (D). Values are expressed as nmol/mg LDL protein and are mean ± SD for triplicate wells from a single experiment representative of three. The difference between the values for cell-containing incubations is not significant ($P > 0.05$ by two-sample *t*-test). Due to the considerable variations in the kinetics and degree of oxidation of TOH-depleted LDL between three individual experiments (see Results), combined mean and SD values for these experiments are not presented.

mouse peritoneal macrophages and human monocytes do not affect their ability to promote LDL oxidation, regardless of the initial concentration of α-TOH in the lipoproteins exposed to oxidative stress.

Effect of α-TOH-enrichment of MPM on superoxide release and copper(II) reduction by cells, and on cellular lipoxygenase activity

To test whether α-TOH loading of MPM affects some cellular functions which might be related to the oxidizing capacity of the cells, we studied the effect of cellular α-TOH level on superoxide release by MPM, reduction of Cu(II) to Cu(I) by the cells and their LOX activity. Control (34 ± 12 pmol α-TOH per mg cell protein) and α-TOH-enriched MPM (26.5 ± 1.1 nmol α-TOH/mg cell protein) released the same amounts of superoxide after 90 min: 0.9 ± 0.2 and 0.9 ± 0.5 nmol superoxide/90 min per 4×10^6 cells, respectively (the values are means ± range; results from two independent experiments). PMA, a potent stimulant for the respiratory burst oxidase of resident macrophages, caused significant increase in superoxide release from MPM, which was linear for at least 90 min (data not shown). This resulted in ≈16.5-fold increase in the amount of superoxide produced by the activated cells during the first 90 min of the respiratory burst in comparison with non-activated MPM. Superoxide release by PMA-stimulated α-TOH-enriched macrophages occurred at the same rate as by stimulated control cells (data not shown), yielding after 90 min of incubation, 14.8 ± 0.6 and 14.9 ± 0.4 nmol superoxide per 4×10^6 cells, respectively. Similarly, there was no significant change in the ability of the cells to reduce Cu(II) after α-TOH enrichment; control and α-TOH-loaded MPM reduced 14.4 ± 1.7

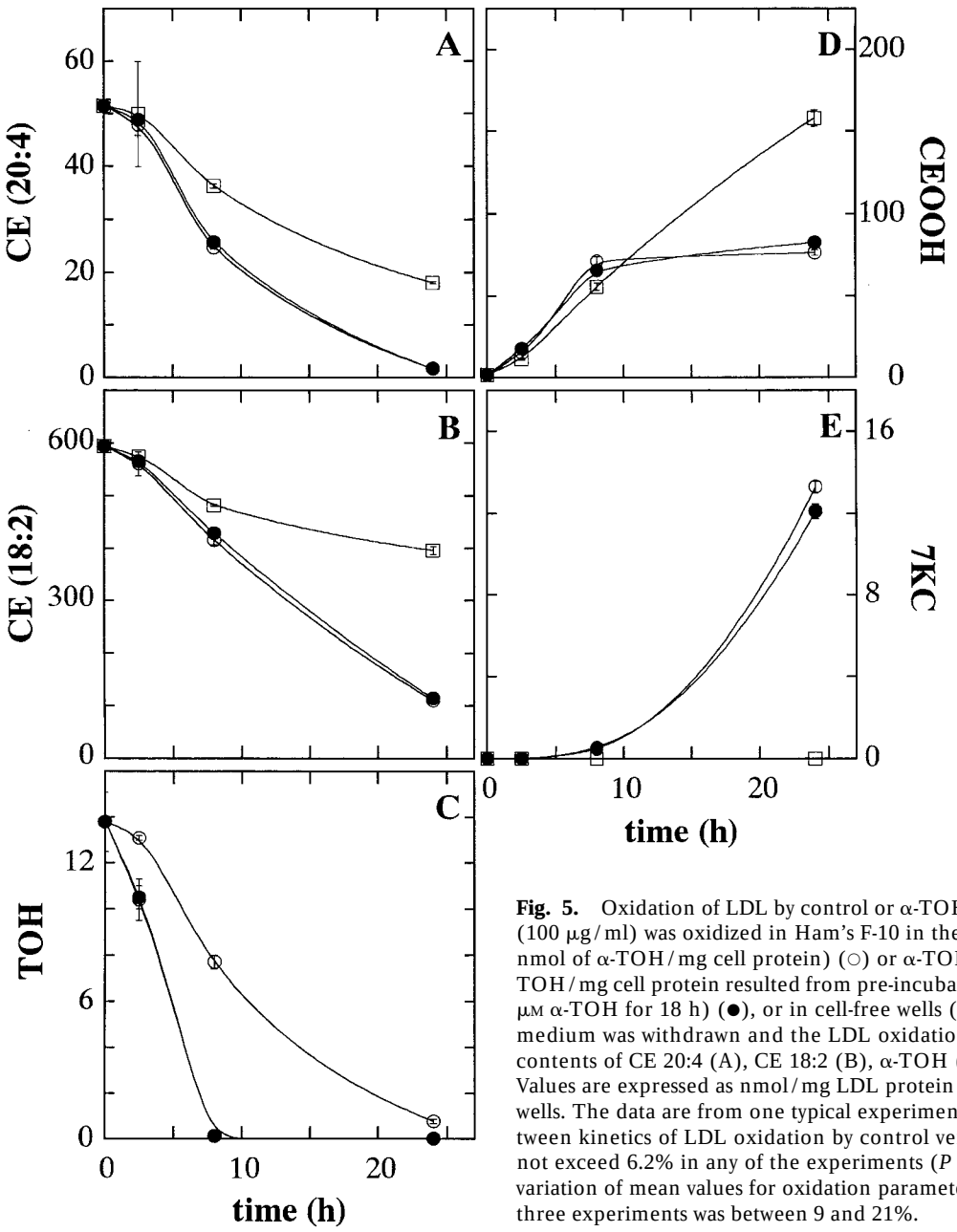

Fig. 5. Oxidation of LDL by control or α-TOH-loaded human monocytes. LDL (100 μg/ml) was oxidized in Ham's F-10 in the presence of control hMON (0.3 nmol of α-TOH/mg cell protein) (○) or α-TOH-enriched hMON (2.96 nmol α-TOH/mg cell protein resulted from pre-incubation of hMON in medium with 23 μM α-TOH for 18 h) (●), or in cell-free wells (□). After the times indicated the medium was withdrawn and the LDL oxidation was assessed by changes in the contents of CE 20:4 (A), CE 18:2 (B), α-TOH (C), CEOOH (D), and 7-KC (E). Values are expressed as nmol/mg LDL protein and are mean ± SD for triplicate wells. The data are from one typical experiment out of three. The difference between kinetics of LDL oxidation by control versus TOH-loaded monocytes did not exceed 6.2% in any of the experiments ($P > 0.05$ by two-sample *t*-test). The variation of mean values for oxidation parameters at any particular time point in three experiments was between 9 and 21%.

and 15.1 ± 0.2 nmol of Cu(II)/90 min per 4×10^6 cells, respectively. Therefore, the rates at which MPM release superoxide radical (under both resting and PMA-activated conditions) and reduce extracellular Cu(II) were not affected by the cellular levels of α-TOH.

In the presence of AA in the culture medium, MPM synthesize 12- and 15-HETEs. The formation of these compounds, catalyzed by cellular LOX, was observed both in the culture medium and in the cellular extract (total amount of 12-HETE and 15-HETE after 1 h incubation, correspondingly, 25.9 ± 1.2 and 10.9 ± 4.3 nmol per mg cell protein). The ratio of 12-HETE to 15-HETE, on average, was 3.5–7 to 1, which is similar to previously reported results (37). Enrichment of MPM with α-TOH resulted in a modest increase in formation of these compounds, both in the cellular extracts (≈1.5-fold) and in the conditioned medium (≈1.4-fold) in two out of three independent experiments (in the third experiment no significant different in HETEs formation by α-TOH-loaded and control macrophages was observed) (data not shown). Generation of the corresponding hydroperoxyeicosatetraenoic acids (HPETEs)

was also observed upon addition of AA to the cells. The percent of 12- plus 15-HPETEs in the total pool of corresponding lipid hydroxide and hydroperoxide was 32.9 ± 2.9 in the cellular extracts (mean ± SD of three independent experiments) and 5.6 ± 1.7 in the media samples. This parameter was not affected by the α-TOH-loading of macrophages. While α-TOH supplementation of MPM led to an overall increase in the activity of LOX to exogenously added substrate, the much lower basal activity, measured in the absence of added AA (≈2.76 ± 0.3 nmol 12- plus 15-HETEs per mg cell protein per 60 min in the cellular extracts and undetectable in the culture medium) was unaffected by α-TOH enrichment (data not shown).

DISCUSSION

While a great body of work exists on the ability of different lipid- and water-soluble antioxidants to affect cell-mediated LDL oxidation, the majority of these studies have concentrated on antioxidants endogenous to lipoprotein particles (14–18) or added extracellularly to the incubation mixture (12, 13). This results in the uncertainty about the location where antioxidants exert their inhibitory action, within the LDL particle, the cell, or in the medium. In this paper we have studied how the manipulation of cellular antioxidant content affects the ability of the cells to oxidize LDL. α-TOH, the most biologically active form of vitamin E (42), has been the antioxidant of major interest, as it is recognized as quantitatively the major lipid-soluble antioxidant in the circulation, and its plasma and tissue levels can be modulated by diet and supplementation. Another important reason to study the effect of cellular α-TOH content on the oxidizing capacity of cells towards LDL is based on the observation that most cultured cells are deficient in vitamin E (17, 43–46), and therefore, such cells might oxidize LDL differently to cells in vivo. We chose mouse peritoneal macrophages as a cellular model, as these cells have been widely used in our, and other, studies of cell-mediated LDL oxidation, and of uptake and degration of such modified lipoproteins by cells (14, 39, 47, 48).

We demonstrated that cellular content of α-TOH can be increased by supplementing the culture medium with the antioxidant. A similar approach has been used by others, and time- and concentration-dependent α-TOH incorporation into the cells has previously been demonstrated for several different cell types (26, 43–45). Our results on the degree of enrichment of MPM with α-TOH in vitro are similar to those previously obtained for α-TOH uptake by cultured human and bovine endothelial cells, J774 macrophages, smooth muscle A7r5 cells, and L1210 lymphoblastic leukemia cells (17, 26, 43, 45, 46, 49) (**Table 2**). Loading human monocytes with α-TOH was less efficient with the method used here, and higher concentrations of α-TOH in the medium were required to achieve a similar degree of α-TOH enrichment of these cells (Table 2).

Almost 88% of α-TOH incorporated into MPM (in absolute amounts) associated with membrane-containing cellular organelles and approximately 11% of α-TOH was present in the cytosol. Similar results were reported by Chan and Tran (26) for endothelial cells. Within the membranous fractions, the highest amounts of α-TOH were found in the plasma membrane (≈41%) for MPM (the present study), and in the fraction containing mitochondria plus lysosomes (≈60%) for endothelial cells (26). This difference could be explained by the different surface area of these cells, and possibly by the different rates of cellular α-TOH metabolism and the use of different methods for subcellular fractionation. In contrast to these cells, in smooth muscle A7r5 cells almost 60% of incorporated α-TOH accumu-

TABLE 2. α-TOH content in different cells after their incubation in α-TOH-supplemented media for the indicated periods of time

Cells	Content of α-TOH in the Loading Medium	Time of Loading	α-TOH in Cells		Reference
			nmol/mg prot.	nmol/10^6 cells	
	μm	*h*			
J774	100	24	19 ± 7	n.a.	17
Smooth muscle cells (A7r5)	50	24	32 ± 1	n.a.	43
Human umbilical cord vein endothelial cells	23	8	n.a.	2.6 ± 0.4	26
Rat hepatocytes	25	3	n.a.	2.0 ± 0.3	45
Baby hamster kidney cell line (BHK-21/c13)	14	96	n.a.	2.9 ± 0.6	46
	21	96	n.a.	5.3 ± 0.3	46
Bovine aortic endothelial cell line (GM 7372A)	100	20	23 ± 4	n.a.	49
Mouse peritoneal macrophages	43	8	n.a.	1.6 ± 0.3	present study
	120	16	26 ± 5	5.1 ± 0.9	
Human monocytes	200	18	19.1 ± 0.2	0.32 ± 0.004	present study

n.a., not available.

lated in the cytosol (43), which was explained by the presence of a cytosolic tocopherol-binding protein in these cells and was attributed to a specific role of α-TOH regulating smooth muscle cell proliferation unrelated to the antioxidant action of the vitamin at the membrane level (43).

Incorporated α-TOH remained stable in MPM for at least 24 h both in the serum-free and LDL-free media whether permissive or non-permissive for oxidation. This indicates that during this time intracellular α-TOH was neither autoxidized under these culture conditions nor was it transferred to the medium in the absence of a suitable acceptor. This differs from a previous study using endothelial cells where about 40% of α-TOH was lost within 24 h after its incorporation (26). We speculate that this loss may have been due to the presence of 20% FCS in the culture medium used in that study and a possible role of albumin and the lipoproteins in fetal calf serum in mediating α-TOH efflux from the cells.

It is believed that cells can promote LDL oxidation via one or more of the following mechanisms: a direct action of cell-derived oxidants on the lipoprotein, maintenance of transition metals in a reduced and therefore highly reactive state by provision of extracellular reductants or by direct reduction of iron and copper ions, introduction of peroxides into LDL by direct action of enzymes (e.g., LOX), or by transfer of cell-derived lipid hydroperoxides to LDL (1, 11, 35). Manipulation of the content of lipid-soluble antioxidants in cells might be expected to modify their capacity to oxidize LDL either by interference with the generation of cellular oxidants, by direct supplementation of lipoproteins with antioxidants, or by interference with the physical interaction between the cell and the lipoprotein. Results presented in this paper demonstrate that enrichment of mouse peritoneal macrophages with α-TOH by their supplementation with relatively physiological concentrations of the antioxidant (in the range normally found in human blood plasma) does not change significantly their oxidizing capacity towards LDL. This suggests that under conditions of this study the oxidative modification of LDL was either not mediated by cell-derived free radical species or that cellular α-TOH was unable to affect their generation (e.g., in case of hydrophilic radicals). We have demonstrated here that loading of MPM with the vitamin did not affect the production of superoxide by the cells whether they were resting or PMA-activated. This differs from the data reported in a recent study on freshly isolated human monocytes (41), in which in vivo oral α-TOH supplementation was used, suggesting that this effect on superoxide generation by monocytes may be indirect. The results presented in the present paper are not sufficient for us to speculate on the involvement of superoxide in cell-promoted oxidation of LDL, although we have previously shown a lack of association between superoxide output and cell-mediated LDL oxidation by MPM or human monocyte-derived macrophages under similar conditions (25, 47). This contradicts recent observations by Deveraj, Li, and Jialal (41) that superoxide radical production by human monocytes correlates with lipoprotein oxidation mediated by the cells, as assessed by the formation of thiobarbituric acid reactive substances (TBARS). However, the TBARS method does not correlate well with more sensitive and direct HPLC methods used to measure indices of cell-mediated LDL oxidation (25), which may explain the discrepancy between the results obtained by these authors (25, 41).

Another possible mechanism by which the manipulation of cellular α-TOH content could affect LDL oxidation might be via supplementation of LDL with the vitamin. We have demonstrated that incubation of α-TOH-enriched MPM with LDL resulted in a transfer of small amounts of α-TOH from the cells to extracellular LDL. This transfer was more obvious in the case when TOH-depleted LDL was present in the culture, when LDL endogenous α-TOH could not obscure determination of the small quantities of the transferred α-TOH. However, this transfer from α-TOH-rich MPM to the lipoprotein did not have significant impact on the rate of cell-mediated LDL oxidation. One of the possible explanations of the lack of effect of α-TOH transferred to the lipoprotein on its susceptibility to oxidation could be that the rate of this process was very low in comparison with α-TOH consumption during the oxidation. The mean α-TOH content of freshly isolated LDL was usually in the range of 8–12 nmol/mg of LDL protein. Under cell-oxidizing conditions in our experiments, α-TOH was consumed by 4 h (Fig. 3), which gives an approximate rate of TOH oxidation of ≈2–3 nmol/mg of LDL per h. The rate of transfer of α-TOH from cells to α-TOH-depleted LDL was ≈0.1 nmol/mg of LDL per h (calculated from a linear increase in α-TOH concentration in the lipoprotein to 1 nmol/mg during 9 h), which is at least 20 times slower. One could speculate that under conditions of facilitated TOH-transfer to LDL (via yet unknown mechanisms) or low radical flux in the system, when the rate of consumption of α-TOH in LDL becomes comparable with that of the α-TOH transfer from cells to LDL, the transferred α-TOH could affect resistance of LDL to oxidation. Lower radical flux in the system could be achieved by, for example, decreasing the cell number per culture and/or concentrations of transition metals in the medium. Our preliminary experiments on LDL oxidation mediated by MPM plated at different density (from 1×10^6 to 4×10^6 cells per culture) have not demonstrated significant

variation in the rate of LDL α-TOH oxidation (data not shown). This might suggest that other factors, e.g., concentrations of transition metals in the medium or intrinsic lipid hydroperoxides in LDL particles, could be of a greater importance in regulation of a radical flux in the system. Our results on the lack of effect of cellular α-TOH status on cell-mediated LDL oxidation also suggest that cellular α-TOH probably does not interfere with the physical interaction between cell and lipoprotein, suggested earlier to be unimportant for the cell-mediated oxidation of LDL (48).

We also examined whether the manipulation of the α-TOH level in MPM affects some other cellular functions that could be related to cell-mediated LDL oxidation (1, 11). We have recently shown that direct cellular reduction of extracellular Fe(III) and Cu(II) may contribute to macrophage-mediated LDL oxidation (35). Loading of macrophages with α-TOH did not affect the ability of the cells to reduce Cu(II). This suggests that α-TOH does not significantly contribute to this process; although it could act as a reductant for Cu(II) in other systems (22, 50). The correlation between the lack of effect of α-TOH supplementation on macrophage-mediated LDL oxidation and Cu(II) reduction is consistent with our suggestion that extracellular copper reduction contributes to cell-mediated oxidation (35).

It has been suggested that another possible mechanism by which cells promote LDL oxidation could be the involvement of cellular lipoxygenases (1, 11). These enzymes catalyze stereospecific incorporation of molecular oxygen into AA (and other polyunsaturated fatty acids) with formation of a range of HPETEs that are further reduced to the corresponding hydroxides. It has been suggested that lipid hydroperoxides could be introduced into LDL (either via direct action of LOX on LDL lipids or transfer of cell-derived LOOH to LDL) (1, 5, 11, 51), and could make LDL more susceptible to oxidation in the presence of transition metals. Under our experimental conditions, MPM in the presence of exogenous AA possess detectable LOX activity, which was indicated by the accumulation of 12- and 15-HETEs in the culture medium and cellular extracts, in agreement with the recent demonstration of the expression of 12/15-LOX gene in mouse resident peritoneal macrophages (37). The majority of LOX-derived HPETEs were apparently reduced intracellularly and secreted in the form of the corresponding hydroxides (HETEs). This was indicated by the different ratio of HPETEs to HETEs in the cellular extracts and in the conditioned medium. Thus, for 12-H(P)ETE, the main products (amongst those analyzed here) synthesized upon addition of AA to MPM, this ratio changed from 1:2 in cells to 1:17 in the medium. Enrichment of MPM with α-TOH resulted in an increase in accumulation of HETEs in both the medium and the cells (on average 40% and 50%, respectively). This indicates that manipulation of cellular α-TOH levels affected the 12/15 LOX-dependent metabolism of exogenously added AA. On the other hand, the very low basal LOX activity assessed by measurement of cellular 12- plus 15-HETEs derived from endogenous substrates appeared to be unaffected by α-TOH supplementation. Together with the observation that cellular α-TOH did not affect the kinetics of LDL oxidation by MPM, these results might question the importance of LOX, or at least its rate-limiting role, in cell-mediated modification of LDL under the conditions of this study. However, this needs to be further investigated using more direct approaches.

The results reported in this paper cannot, however, exclude the possibility of a different effect of α-TOH under other conditions. Thus, Suzukawa and colleagues (17) recently demonstrated that loading of mouse macrophage cell line J774 with high concentrations of vitamin E (1 mM in the supplementation medium) resulted in a reduced ability of the cells to oxidize LDL. However, in the same study, when cellular α-TOH levels were similar to those achieved here (19 nmol/mg cell protein after pretreatment with 100 μM vitamin E) the difference in accumulation of oxidation products in the medium (measured as TBARS) was small when compared to control cells. The difference between our observations and those obtained by these authors using very high concentrations of α-TOH may possibly be explained by the effect of α-TOH on the J774 proliferation (not yet known), as shown for other cells (52, 53), as well as by the use of a less specific (TBARS) assay to measure LDL oxidation. Our results on the lack of the effect of enrichment of human monocytes with α-TOH on their oxidizing capacity towards lipoprotein differ from the data reported for these cells in (41). This discrepancy could possibly be explained by the different methods used for enrichment of the cells with α-TOH. In the previous study (41) the oral supplementation may have indirectly altered the monocyte population in vivo leading to an altered capacity to oxidize LDL not directly related to cellular α-TOH status.

The absence of an effect of cellular α-TOH on cell-mediated LDL oxidation demonstrated here does not diminish the antiatherogenic potential of α-TOH, demonstrated in numerous epidemiological and animal studies (1, 5–8). The reported beneficial effects of α-TOH related to atherosclerosis, which are exerted at the cellular level, include its inhibition of adhesion of monocyte to endothelial cells (41, 54), stimulation of endothelial cells proliferation (53) after pretreatment with α-TOH, increased resistance of cultured endothelial cells to the cytotoxic effects of oxidized LDL (49), slower rates of lipid peroxidation in cells loaded with

polyunsaturated fatty acids (55), and inhibition of proliferation of vascular smooth muscle cells (52). Preincubation of endothelial cells with α-TOH has been demonstrated to potentiate prostacylin release, which, in turn, has vasodilating and anti-platelet-aggregating properties (56). Recently it has been shown that α-TOH supplementation can result in a significant decrease in release of potentially atherogenic cytokine interleukin-1β from monocytes (41) or in protection of vascular endothelium from oxidized LDL-mediated dysfunction (57).

In conclusion, the results presented in this paper show that cells with different levels of α-TOH, under conditions used here, oxidize LDL at the similar rate. Thus, vitamin E content of mouse peritoneal macrophages or human monocytes does not appear to affect their propensity to oxidatively modify LDL. Although the data reported here do not exclude the possible involvement of such cellular functions as superoxide release, transition metal reduction, and LOX activity in cell-mediated lipoprotein oxidation, they suggest that cell-mediated oxidation of LDL under conditions of this study is not likely to be dependent on the cell-derived free radicals or that cellular α-TOH is unable to control their generation. Together with the results from numerous studies that demonstrated the effect of content of LDL endogenous vitamin E on the lipoprotein oxidation (14–18), our results on the lack of effect of cellular α-TOH on this process might suggest that α-TOH content of LDL, but not that of the macrophages, is responsible for inhibition (or promotion) of LDL oxidation.

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