Free Rad. Res., Vol. 26, pp. 351–362
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Toxicity of Polyunsaturated Fatty Acid Esters for Human Monocyte-Macrophages: The Anomalous Behaviour of Cholesteryl Linolenate

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Accepted by Prof. B. Halliwell

(Received 17 October 1996; In revised form 4 November 1996)

We have investigated the toxicity to human monocyte-macrophages, and susceptibility to oxidation, of different individual dietary fatty acids in cholesterol esters and triglycerides, added to the cell cultures as coacervates with bovine serum albumin. Toxicity was assessed using release of radioactivity from cells preloaded with tritiated adenine. Lipid oxidation was measured by gas chromatography (GC).

The triglycerides showed a direct relationship between toxicity and increasing unsaturation, which in turn correlated with increasing susceptibility to oxidation. Triolein (18:1; ω-9) and trilinolein (18:2; ω-6) were non-toxic. Trilinolenin (18:3; ω-3) was toxic only after prolonged incubation. Triarachidonin (20:4; ω-6), trieicosapentaenoin (20:5; ω-3) and tridocosahexaenoin (22:6; ω-3) were profoundly and rapidly toxic. There was a similar relationship between toxicity and increasing unsaturation for most of the cholesterol esters, but cholesteryl linolenate was apparently anomalous, being non-toxic in spite of possessing three double bonds and being extensively oxidised. Probucol and DL-α-tocopherol conferred protection against the toxicity of cholesteryl arachidonate and triarachidonin.

The oxidation in these experiments was largely independent of the presence of cells. GC indicated that formation of 7-oxysterols might contribute to the toxicity of cholesteryl linoleate. The toxicity of triglycerides suggests that polyunsaturated fatty acid peroxidation products are also toxic. Possible mechanisms of cytotoxicity and relevance to atherosclerosis are discussed.

Keywords: Cytotoxicity, oxidation, polyunsaturated fatty acid esters, linolenate, monocyte-macrophages (human), atherosclerosis

Abbreviations: BSA, bovine serum albumin; CA, cholesteryl arachidonate; CD, cholesteryl docosahexaenoate; CE, cholesteryl eicosapentaenoate; CL, cholesteryl linoleate; CLN, cholesteryl linolenate; CO, cholesteryl oleate; GC, gas chromatography; HMM, human monocyte-macrophages; 7β-OH-CHOL, 7β-hydroxycholesterol; LDL, low-density lipoprotein; LPD-FCS, lipoprotein-deficient foetal calf serum; MPM, mouse peritoneal macrophages; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; TA, triarachidonin; TD, tridocosahexaenoin; TE, trieicosapentaenoin; TL, trilinolein; TLN, trilinolenin; TO, triolein.

INTRODUCTION

Considerable evidence now supports the idea that peroxidation of lipids and lipoproteins occurs during the pathogenesis of atherosclerosis.[1,2]

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Oxidised lipids are present in human atherosclerotic lesions at all stages of development.[3] Polyunsaturated lipids (e.g., linoleate) are depleted relative to oleate in human foam-cell-rich lesions, when compared with plasma low density lipoprotein (LDL), normal artery wall or whole plasma.[3]

The characteristic lipid-rich core of the advanced lesion contains debris of macrophages.[4] The death of macrophage foam cells therefore appears to be a factor in progression of the human atherosclerotic lesion. The cause is uncertain, but one possibility is toxicity of oxidised LDL (oxLDL). Toxicity of oxLDL *in vitro*, including LDL oxidised by macrophages, was shown some years ago,[5–7] but its cytotoxic effects on mouse peritoneal macrophages (MPM) were demonstrated more recently.[8] A coacervate with bovine serum albumin (BSA) of one of the constituents of LDL, cholesteryl linoleate, was also toxic to MPM.[9] This toxicity, and that of oxLDL, was partially inhibited by the antioxidant α-tocopherol.[8,9] Peroxidation of the linoleate moiety appeared to be responsible for initiating the toxicity of the coacervate, because cholesteryl oleate/BSA, which is not peroxidised by MPM, was not toxic.[9]

We have shown that oxLDL is toxic also to human monocyte-macrophages (HMM) in a concentration- and time-dependent fashion,[10] dependent upon the degree of oxidation of the LDL.[11] Oxysterols, including 7β-hydroxycholesterol, which are found in oxLDL, in oxidised cholesteryl linoleate and in the human lesion, are also toxic to HMM *in vitro*.[12]

The composition of plasma LDL depends largely upon the diet. The aim of the present study was to investigate the contribution to oxLDL toxicity of cholesterol esters and triglycerides of different dietary fatty acids, incubated with HMM. Pure lipids, all natural constituents of LDL, were prepared individually as coacervates with BSA. These "artificial lipoprotein" particles are taken up by macrophages by a process involving scavenger receptor activity[13] which ultrastructurally resembles phagocytosis.[14] We have assessed toxicity in conjunction with lipid oxidation in the medium and the effects of the lipophilic antioxidants DL-α-tocopherol and probucol, in the HMM.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated all chemicals and biochemicals were obtained from the Sigma Chemical Co. Ltd (Poole, Dorset, U.K.) and were of the highest grade of purity available. Organic solvents were purchased from Fisons or BDH and were of analytical grade or better.

Isolation of Human Monocyte-Macrophages

HMM were isolated from peripheral blood from healthy volunteers, as described previously.[11] Blood diluted with phosphate-buffered saline (PBS) was layered onto Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 300*g*. The mixed mononuclear cells were removed from the interface and plated in 24-well dishes (Falcon; Becton Dickinson Labware, Franklin Lakes NJ, USA) at a density of 3×10^6 cells/ ml in Macrophage-SFM medium (Life Technologies Ltd; Paisley, Scotland, U.K.; 1 ml/well). After 1 h, non-adherent cells were removed, leaving adherent HMM ($0.5–1.0 \times 10^6$ cells/ well; > 90% purity by Giemsa staining). Prior to addition of coacervates, the medium was replaced with RPMI 1640 + 10% heat-inactivated lipoprotein-deficient foetal calf serum (LPD-FCS; 1 ml/well).

Preparation of Coacervates of Cholesterol Esters and Triglycerides

Coacervates were prepared using a modification of the method of Werb and Cohn.[15] Cholesterol ester was dissolved in 0.5 ml acetone and the solution added to 4 ml BSA solution (10 mg BSA/

ml PBS) with vortexing and sonication for 1 min. Acetone was evaporated under nitrogen and the coacervates so produced used immediately. When preparing coacervates of triglycerides, the weight of triglyceride was adjusted such that the concentrations of fatty acid was the same for the triglycerides and the cholesterol esters. Coacervates were added to cultures at three volumes; 10, 50 and 100 µl/ml, corresponding to final concentrations of fatty acid in the medium of 88, 440 and 880 µM, respectively. The concentration of fatty acid was the same in incubations with cholesterol esters (one fatty acid chain) and triglycerides (three fatty acid chains), and all lipid concentrations quoted subsequently refer to the fatty acid chain. Where appropriate, DL-α-tocopherol (200 µM final concentration) and probucol (50 µM final concentration) were incorporated into the coacervates.

Synthesis of Cholesterol Esters of Eicosapentaenoic and Docosahexaenoic Acids

These cholesterol esters were not commercially available. Fatty acid (10 mg) was treated with oxalyl chloride (2 ml; Aldrich), at room temperature, in the dark under argon, for 1 h. Excess oxalyl chloride was evaporated under argon, leaving the fatty acid chloride as an oil. Cholesterol (35 mg; BDH Ltd) dissolved in dichloromethane (2 ml) was added to the fatty acid chloride, mixed and allowed to react at room temperature in the dark under argon overnight. The dichloromethane was evaporated under argon, and the mixture of cholesterol ester and excess cholesterol subjected to preparative thin layer chromatography (TLC) to purify the cholesterol ester. TLC plates (Whatman PK6F) were 20 cm × 20 cm and glass backed, coated with a 1 mm thick layer of silica gel (pore size 60 Å) containing fluorescent indicator (254 nm). The plates were pre-eluted in ethyl acetate and air-dried. The reaction products (dissolved in dichloromethane) were then applied and the plates eluted using dichloromethane. The bands were visualized with UV light. The cholesterol ester band had an R_f of ca. 0.9 (the same as a standard cholesterol ester, cholesteryl oleate) and was well separated from free cholesterol (R_f of ca. 0.1). It was scraped off, and the cholesterol ester recovered using dichloromethane which was evaporated under argon, leaving the cholesterol ester as an oil, which varied from pale yellow to colourless. Overall yields of cholesterol esters were 49–68%.

Cytotoxicity Assay

Cytotoxicity was determined using a method described previously.[11] Cells were incubated with 1 µCi/ ml [8-^{3}H]-adenine (specific activity 24 Ci/ mmole; Amersham Radiochemicals Ltd., Aylesbury, Bucks) for 1 h at 37°C. Unincorporated adenine was removed by rinsing with PBS, and replacing with RPMI 1640 + 10% LPD-FCS. At time points, the medium was removed and sampled. Intracellular radioactivity was extracted by lysing pooled detached cells after centrifugation and adherent cells, with 1% Triton X-100 (1 ml). 200 µl samples of medium and lysate were counted by liquid scintillation counting (disintegrations per minute).

Extraction and Analysis of Lipids by Gas Chromatography

Lipids were extracted from the medium and processed for GC as described previously.[16] Internal standards (*n*-heptadecanoic acid, coprostane and 5α-cholestane) were added followed by Bligh and Dyer extraction, sodium borohydride reduction, saponification and derivatisation to methyl esters and trimethylsilyl ethers. Analysis by GC was performed as previously described,[17] using a 30 m DB-1 fused silica capillary column (0.32 mm internal diameter, 0.1 µm thickness; J and W Scientific, Folsom, CA, USA). Quantitation was performed using peak areas, measured electronically using an integrator, relative to internal standards.

Statistics

Statistical analyses were carried out using unpaired Students' *t*-tests with Microsoft Excel™ software for PC. Results were considered to be significantly different at $P \leq 0.05$. All experiments were carried out a minimum of three times, in duplicate or triplicate wells.

RESULTS

Concentrations of fatty acids were the same with cholesterol esters (one fatty acid chain) and triglycerides (three fatty acid chains); all lipid concentrations quoted refer to the fatty acid.

Cytotoxicity of Cholesterol Esters

Radioactivity leakage from cells preloaded with tritiated adenine is generally considered to reflect impaired membrane integrity,[18] correlating with other methods of determining cell damage including leakage of lactate dehydrogenase, but being the most sensitive.[9,10]

Cholesteryl oleate (18:1; CO) was not toxic at any of the concentrations assessed up to and including 48 h (Fig. 1A). Cholesteryl linoleate (18:2; CL) produced a time- and concentration-dependent toxicity in HMM. (Fig. 1B). In contrast, cholesteryl linolenate (18:3; CLN) was not cytotoxic. Indeed, exposure resulted in a slightly lower leakage of radioactivity than controls (Fig. 1C), which was statistically significant at 48 h exposure to 88 and 440 μM of the coacervate. Cholesteryl arachidonate (20:4; CA) produced striking toxicity (Fig 1D). 440 and 880 μM CA produced near-total leakage at 48 h, with some damage at 24 h. 88 μM CA was significantly toxic only after 48 h (Fig. 1D). Cholesteryl eicosapentaenoate (20:5; CE) caused significant toxicity at all concentrations from 24 h onwards with near total leakage at 48 h exposure to the higher concentration (Fig. 1E). Cholesteryl docosahexaenoate (22:6;CD) produced similar toxicity to CE (Fig. 1F).

Cytotoxicity of Triglycerides

Exposure of cells to triolein (18:1; TO) did not cause detectable adverse effects after 48 h at any concentration (Fig. 2A). Indeed, at 880 μM, there was a small but significant diminution of leakage at 24 and 48 h compared with no addition controls. Trilinolein (18:2; TL), at up to 880 μM was also innocuous over 48 h. (Fig. 2B). Trilinolenin (18:3; TLN) produced toxicity at 48 h only at 880 μM (Fig. 2C). Indeed, after 48 h exposure to 88 μM TLN, there was a significant reduction in total radioactivity leakage compared to controls. In contrast, triarachidonin (20:4; TA), at 88–880 μM, caused more toxicity at 24 h (Fig. 2D) than CA (Fig. 1D). Trieicosapentaenoin (20:5;TE) and tridocosahexaenoin (22:6;TD) were strikingly and similarly toxic even at 88 μM (Figs. 2E and 2F).

Lipid Oxidation

Most oxidation in the medium was independent of the presence of cells. CO (18:1) showed little oxidation (data not shown) in accord with previous data.[16] In contrast, CL (18:2) was rapidly depleted to ca. 38% of initial values by 24 h (Fig. 3A). At 24 h, there was a 1.5-fold enhancement in the formation of 7β-hydroxycholesterol from CL in the presence of cells (Fig. 3A). CLN (18:3) was also oxidised, but little 7β-hydroxycholesterol was formed (Fig. 3B). Depletion of linolenate was comparable to that of linoleate, with loss near-maximal at 24 h. Although arachidonate (20:4) was depleted to ca. 30% of initial levels at 24 h, the production of 7β-hydroxycholesterol was lower than for linoleate (Figure 3C). Depletion of fatty acid from both CE and CD was rapid and profound (to ca. 13% and 9% at 24 h, for CE and CD respectively); there was little formation of 7β-hydroxycholesterol (Figs 3D and 3E).

TO (18:1) was only modestly depleted. For 440 μM TO, approximately 60% of the 18:1 remained at 24 h. TA (20:4) was depleted as early as 2 h to 71% of initial levels and by 6 h to

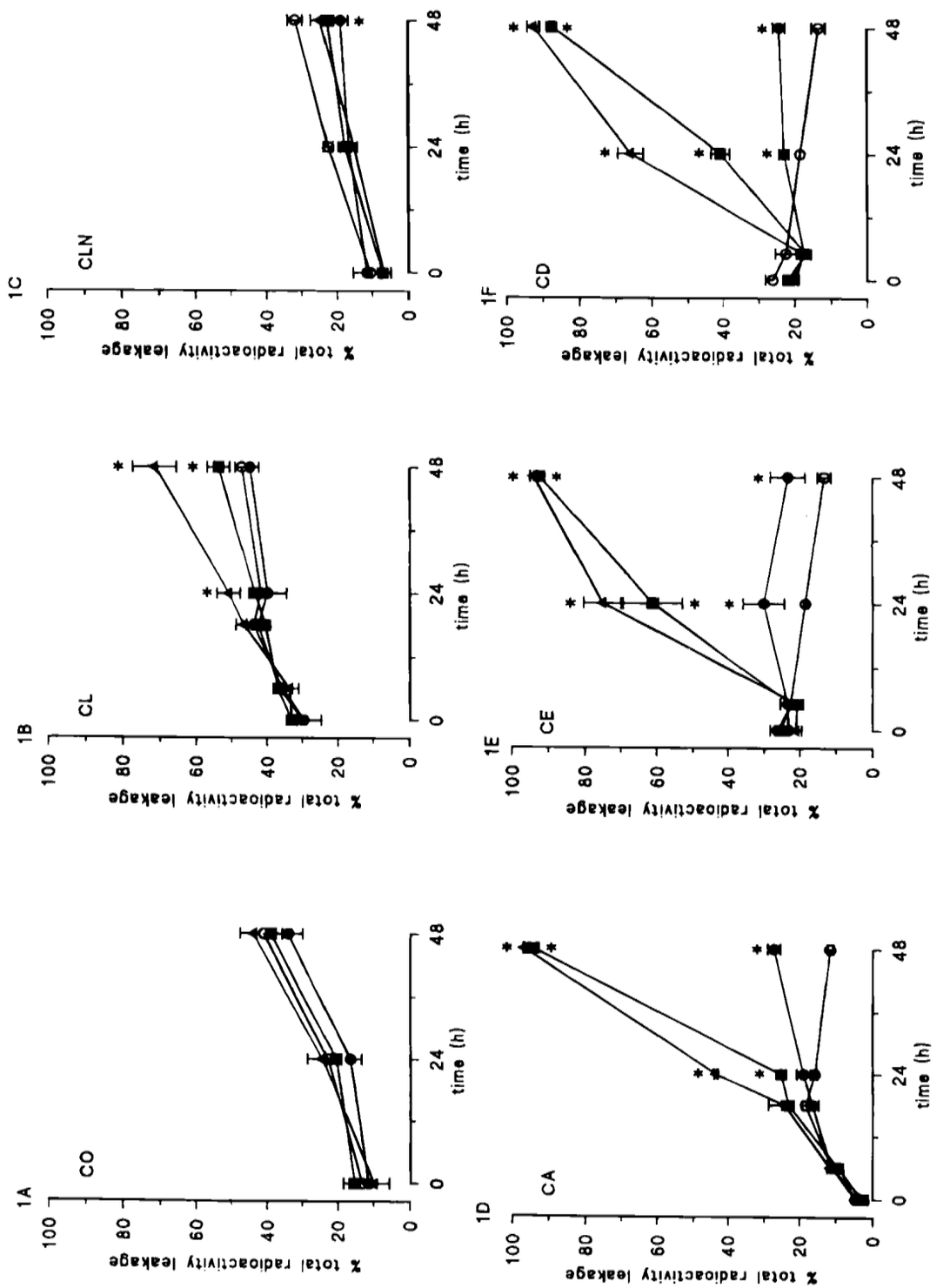

FIGURE 1 *Toxicity of cholesterol esters.* HMM were incubated as no additions controls (open circles) or with 88 (closed circles), 440 (closed squares) and 880 μM (closed triangles) fatty acid as cholesterol ester coacervate in RPM1/ 10% LPD-FCS, for up to 48 h: CO (**A**), CL (**B**), CLN (**C**), CA (**D**), CE (**E**) and CD (**F**). Results represent the mean percentage of total radioactivity leaked into the medium ± SD from one experiment typical of three, in triplicate wells. *Significantly different from controls using unpaired *t*-tests at $P \leq 0.05$.

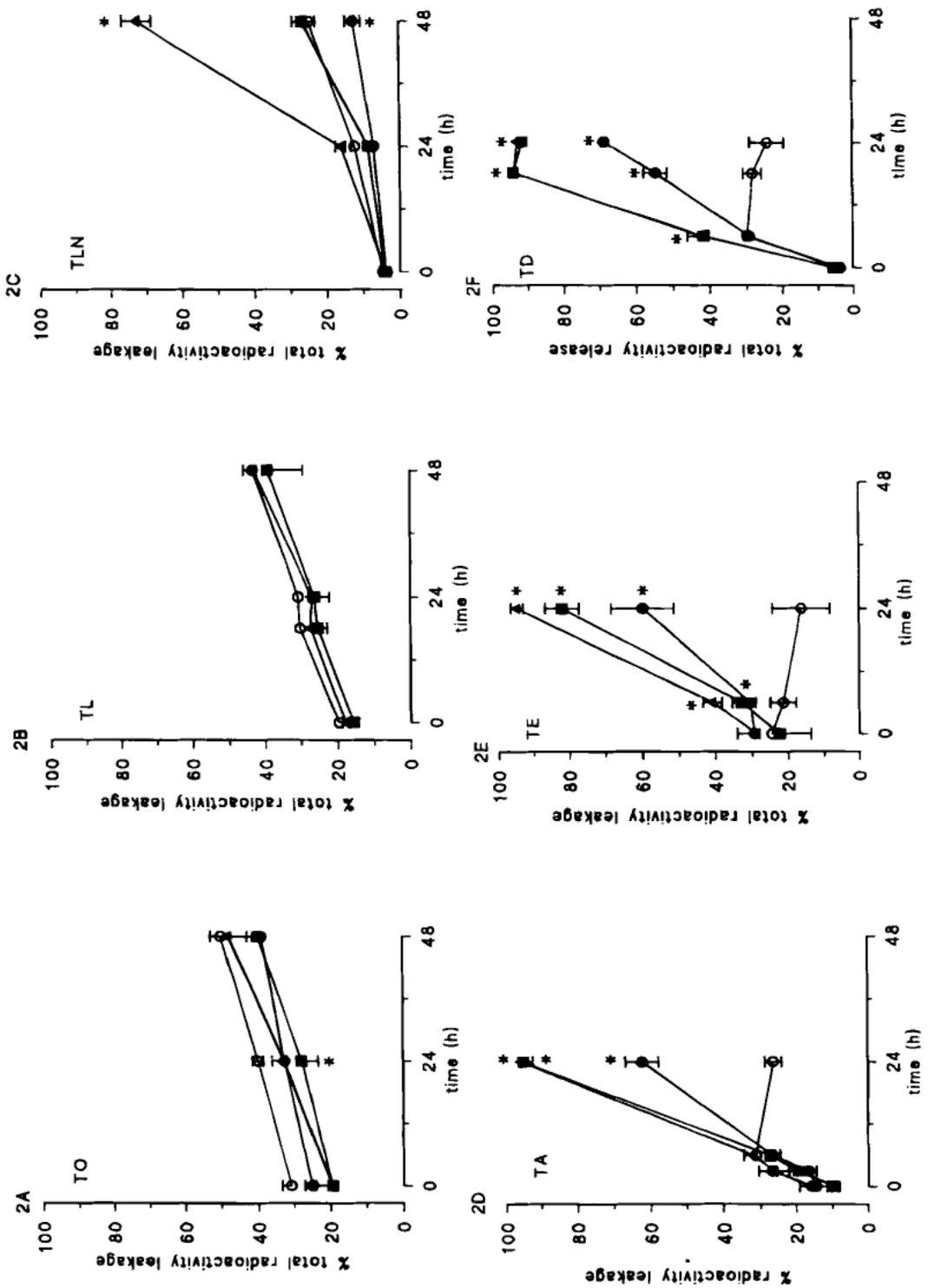

FIGURE 2 *Toxicity of triglycerides.* HMM were incubated as no additions controls (open circles) or with 88 (closed circles), 440 (closed squares) and 880 μM (closed triangles) fatty acid as coacervates of triglycerides, for up to 48 h: TO (**A**), TL (**B**), TLN (**C**), TA (**D**), TE (**E**) and TD (**F**). Results represent the mean percentage of total radioactivity leaked into the medium ± SD from one experiment typical of three, in triplicate wells. *Significantly different from controls using unpaired *t*-tests at $P \leq 0.05$.

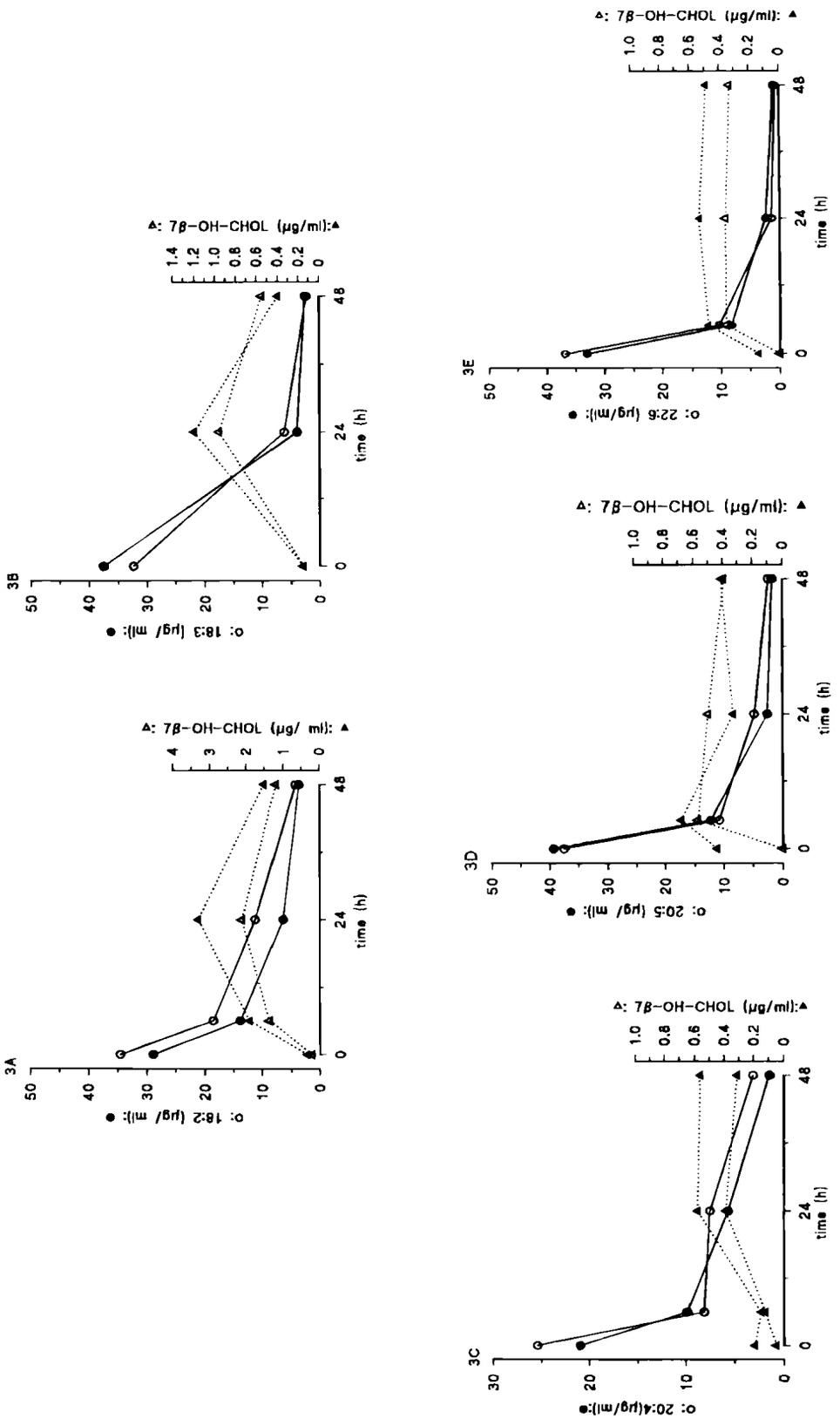

FIGURE 3 *PUFA depletion and formation of 7β-hydroxycholesterol* HMM were exposed to 440 µM CL (**A**), CLN (**B**), CA (**C**), CE (**D**) and CD(**E**) for up to 48 h (closed symbols). Depletion of fatty acid (solid lines) and production of 7β-hydroxycholesterol (broken lines) were measured using GC. Parallel incubations were carried out in the absence of cells (open symbols). The results shown are from one experiment typical of three.

12%. Depletion of 22:6 in TD was even more profound; after 24 h, levels were 3–5% of initial values.

Protection with Antioxidants Against Toxicity

After 24 h exposure, there was enhanced radioactivity leakage due to CA (33.0 ± 7.3% total leakage) compared to controls (16.3 ± 1.0%) which was inhibited by incorporating 200 μM DL-α-tocopherol (11.5 ± 0.7%) or 50 μM probucol (15.7 ± 1.9%) in the coacervates. The leakage due to TA (39.9 ± 2.0%) compared with controls (4.1 ± 0.2%) at 24 h, was also inhibited by DL-α-tocopherol (15.7 ± 1.3%) or probucol (10.0 ± 0.4%). These results represent the mean percentage of total radioactivity leaked ± SD from one experiment typical of three, in triplicate wells. In both sets of experiments, neither α-tocopherol nor probucol, at the same concentrations, affected cell membrane integrity when presented as coacervates in the absence of CA or TA (data not shown).

DISCUSSION

Mode of Oxidation

The analytical method used measures fatty acid species, cholesterol and oxysterols as total free plus esterified forms, as a saponification step is included (see Materials and Methods). Measurement of cellular lipid was not performed, as fresh HMMs accumulate very little intracellular lipid.[16] The results indicate that lipids containing certain PUFAs are oxidised in the culture medium. Cell-mediated oxidation of CL coacervates can be accomplished by HMM under different culture conditions;[16] the apparent lack of cell-mediated oxidation in this study is probably due to the much lower cell numbers.[16] Small contributions of cell-mediated oxidation may be inconspicuous in the strong background of non-biological oxidation. The GC traces were all qualitatively the same in the presence or absence of cells, suggesting that the chemistry was similar in both cases, and that peroxidation is the main cause of depletion rather than β-oxidation. The resistance of oleate to oxidation, compared with PUFA esters, is consistent with this view, since oleate is a good substrate for β-oxidation.

Relationship Between Toxicity and Oxidation

Toxicity of the triglycerides increased with increasing unsaturation, TO = TL < TLN < TA < TE = TD. For cholesterol esters, the order was CLN ≤ CO < CL < CA < CE = CD. The results with CLN are surprising; although it is peroxidised, it appears to be even less toxic than CO. Minor peaks were visible on the GC traces at retention times where hydroxypolyunsaturated fatty acids would be expected to elute, but at no point in the time course of any of the lipids did significant buildup of such peaks occur, suggesting that the hydroxypolyunsaturated fatty acids or their parent hydroperoxy forms were decomposed or catabolised rapidly. Catabolism is unlikely since the GC traces were similar in the presence and absence of cells. Efficient catabolism of hydroperoxides of CLN in comparison to the hydroperoxide of other PUFAs is unlikely to explain the lack of toxicity of CLN, as all the PUFA esters behave similarly in this respect as analysed by GC.

The lack of toxicity of the monounsaturated oleate, as either the triglyceride or cholesterol ester, is probably due to its much lower susceptibility to peroxidation. Hydrogen atoms can be abstracted from a bisallylic methylene group of a PUFA chain (not present in oleate) forming a pentadienyl radical, which picks up oxygen to form a peroxyl radical.

The findings suggest that in this model peroxidation is necessary but not alone sufficient to produce toxicity. The various oxidation products peculiar to each individual lipid species would appear to be important in toxicity.

The Toxic Species

Candidate toxic agents include oxysterols, lipid peroxides, and radicals and aldehydes formed from the decomposition of lipid peroxides, all of which could damage the cell. Oxysterols found in oxidised LDL include 7α- and 7β- hydroxycholesterols, 7-ketocholesterol, and 7α- and 7β- hydro-peroxycholesterols. 7β- Hydroxycholesterol was detected by GC (Fig. 3). 7α-Hydroxycholes-terol levels are likely to be approximately 30% of 7β-hydroxycholesterol, judging from previous studies of CL.[16] The 7α-hydroxycholesterol peak on GC is usually obscured by the cholesterol peak if the latter is sufficiently large and quantitation was not attempted.

The sodium borohydride reduction step also reduces any aldehydes and ketones to alcohols. 7-Hydroxycholesterols detected could derive from 7-hydroperoxycholesterols and 7-ketocholesterol present in the original lipid extract. Previous studies[16] suggest that approximately half of the 7β-hydroxycholesterol derives from 7-ketocholesterol. 7-Hydroperoxycholesterol is toxic to cultured fibroblasts.[19] 7-Oxysterols are formed by hydrogen atom abstraction by a PUFA-derived peroxyl or pentadienyl radical from the 7-position of cholesterol,[20] giving a resonance-stabilised allylic radical, which picks up oxygen producing 7-oxysterols.[17]

Although 7-oxysterols are toxic to HMM,[12] and presumably contribute to the toxicity of cholesterol esters, PUFA-derived species in triglycerides were capable of even greater toxicity, exemplified by TA, TE, TD and TLN. CA produces less 7β-hydroxycholesterol than does CL, suggesting that the toxicity of CA may be due more to peroxidation products of the fatty acid moiety than to oxysterols. CLN was non-toxic and did not produce much 7β-hydroxycholesterol. However, cholesterol levels do fall appreciably for CA and CLN implying that more highly oxidised forms of cholesterol are produced, but escape detection. We cannot exclude the possibility of the formation of a toxic oxidation product of glycerol, which might account for the toxicity of the triglycerides. The lack of toxicity of TO and TL, however, argues against the existence of such a product, and also implies that the modest toxicity of CL is due predominantly to 7-oxysterols. Further, exposure to cholesterol alone does not result in toxicity to HMM.[12]

The absence of toxicity, and even a degree of protection, due to CLN is undoubtedly the most surprising result of this study, particularly as CLN was oxidised. Linolenic acid is an ω-3 fatty acid, whereas arachidonate and linoleate are both ω-6. This positional difference in the double bonds might be involved in differences in toxicity. Linolenic acid oxidation would produce different peroxides and aldehydes than would arachidonate and linoleate. For example, 4-hydroxynonenal and hexanal, which are decomposition products of peroxides of ω-6 fatty acids, would be unlikely to be produced from linolenate peroxides which, like other ω-3 fatty acids, would give propanal and 4-hydroxyhexenal.[21] 4-Hydroxynonenal is more toxic to HMM than 4-hydroxyhexenal, which is in turn much more toxic than hexanal.[22] Malondialdehyde, derived from PUFAs with three or more double bonds, is much less toxic.[22] It might, therefore, be expected that ω-3 fatty acid esters would be less toxic than the ω-6, but the amounts of individual aldehydes produced under particular conditions would naturally be important. TLN is, however, toxic, albeit only after 48 h incubation. This implies that more toxic oxidation products of linolenate are formed from the triglyceride than from the cholesterol ester. This is conceivable since in each triglyceride molecule there are three polyunsaturated fatty acid chains in close proximity and so intramolecular reactions are possible, whereas the cholesterol ester only has one fatty acid chain.

Protection with Lipophilic Antioxidants

Both probucol and DL-α-tocopherol protect LDL from oxidation and reduce its subsequent toxicity to HMM *in vitro*.[11] Probucol also inhibits

lipid core formation in vivo.[23] Mean plasma α-tocopherol levels correlate inversely with the incidence of ischaemic heart disease in European populations[24] and α-tocopherol supplements may protect against the disease.[25] The results of this study are consistent with these observations. DL-α-tocopherol and probucol protected against the toxicity of CA and TA. DL-α-tocopherol and probucol also diminish oxidation of LDL by HMM[26] and by copper ions[11] and decrease oxidation of CL by HMM.[16] The mechanism of protection is not clear. There are two obvious possibilities. First, lipid oxidation may be delayed; secondly, direct protection may be conferred against the peroxidation products. These mechanisms might be complementary.

Relevance to Human Atherosclerosis

This culture model constitutes a rapid means of producing some of the changes found in arterial lesions, including: 1. Depletion of oxidisable lipids such as arachidonate and linoleate; 2. Death of macrophage foam cells; 3. Beneficial effects of lipophilic antioxidants. The death of the foam cells within the lesion may contribute most to production of the acellular core.[4] This may even be the most important stage of progression of the disease, occurring as it does mainly in those populations that suffer the greatest morbidity and mortality.[27]

It is possible that, within the lesion, linoleate and arachidonate contribute to LDL toxicity; linoleate is the most abundant fatty acid in LDL (ca. 2000 nmol/ mg LDL protein); arachidonate, present at approximately 300 nmol/ mg LDL protein,[28] is more toxic.

Linolenate is present in plasma LDL normally at about 10% of the level of arachidonate.[29] In view of the apparent protective nature of CLN, and the delayed toxicity of TLN, it is possible that linolenate in diet may not be harmful in this context. The apparently anomalous behaviour of CLN is of particular interest, especially in view of the fact that a Mediterranean diet rich in linolenate may contribute to the secondary prevention of ischaemic heart disease.[30]

The profound toxicity, and susceptibility to oxidation, of eicosapentaenoic and docosahexaenoic acids, as triglycerides or cholesterol esters, suggest that dietary fish oils might have some undesirable effects. Fish oil supplementation renders LDL more susceptible to oxidation.[31] Eicosapentaenoic and docosahexaenoic acids are normally present in LDL at only about 4% of the level of linoleate but can increase to 15% on dietary supplementation.[31]

The concentrations of esterified fatty acids used in these experiments are much higher than in human plasma. However, lipid concentrations in arterial lesions are significantly higher than in plasma, e.g. for cholesterol 6–20 fold higher (30–100 mM in lesions) and linoleate is in the range 7–21 mM.[32]

CONCLUSION

The results show that the oxidation of some dietary polyunsaturated fatty acids may cause toxicity to macrophages *in vivo*. They suggest that too much polyunsaturated fatty acid in the diet may be harmful. It might be that a diet low in fat, with more monounsaturates than polyunsaturates, would be beneficial, especially if supplemented with α-tocopherol. In spite of being susceptible to oxidation, cholesteryl linolenate is not toxic. The mechanism of this anomaly is unclear and merits investigation.

Acknowledgements

We thank the UK Ministry of Agriculture, Fisheries and Food for support for SJH, KLHC and NSL and the British Heart Foundation for supporting CV, RH and CEM. We are also grateful to Prof. Michael Oliver for discussion and Mrs Virginia Mullins for secretarial assistance.

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