Radical-induced lipoprotein and plasma lipid oxidation in normal and apolipoprotein E gene knockout (apoE$^{-/-}$) mice: apoE$^{-/-}$ mouse as a model for testing the role of tocopherol-mediated peroxidation in atherogenesis

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Abstract Exposure of plasma from apolipoprotein E gene knockout (apoE$^{-/-}$) and control (CBA or C57BL/6J) mice plasma to a constant rate of aqueous peroxyl radicals (ROO$^•$) resulted in the depletion of ascorbate, urate and α-tocopherol (α-TOH), with substantial and little lipid peroxidation, respectively. α-TOH levels were 3-times higher in plasma from apoE$^{-/-}$ than control mice and its addition enhanced the oxidizability of control mouse plasma. In apoE$^{-/-}$ mouse plasma, α-TOH was associated primarily with very low density lipoprotein (VLDL), whereas in plasma from control mice, the vitamin was located largely in high density lipoproteins. Oxidation of isolated lipoproteins by ROO$^•$ resulted in the accumulation of lipid hydroperoxides to an extent that reflected the plasma concentration and α-TOH content of the different lipoprotein fractions. Oxidation of 'plasma' reconstituted from components of apoE$^{-/-}$ mice and/or human plasma showed that human and apoE$^{-/-}$ mouse lipoproteins peroxidized with similar kinetics, although the initiation of lipid peroxidation was greater in the presence of mouse than human lipoprotein-deficient plasma. Also, the chain length of lipid peroxidation in apoE$^{-/-}$ mouse plasma after ascorbate depletion appeared to be independent of the rate of ROO$^•$ generation. Together, these results show that the ROO$^•$ induced peroxidation of plasma lipoproteins in atherogenesis-susceptible apoE$^{-/-}$ mice exhibits some, though not all, features of tocopherol-mediated peroxidation (TMP). Therefore, apoE$^{-/-}$ mice may represent a suitable animal model to test a role for TMP in atherogenesis and the prevention of this disease by anti-TMP agents.—**Neuzil, J., J. K. Christison, E. Iheanacho, J-C. Fragonas, V. Zammit, N. H. Hunt, and R. Stocker.** Radical-induced lipoprotein and plasma lipid oxidation in normal and apolipoprotein E gene knockout (apoE$^{-/-}$) mice: apoE$^{-/-}$ mouse as a model for testing the role of tocopherol-mediated peroxidation in atherogenesis. *J. Lipid Res.* 1998. **39:** 354–368.

Supplementary key words antioxidants • atherosclerosis • lipid peroxidation • vitamin E

Oxidative modification of low density lipoprotein (LDL) has been implicated in the early stages of atherogenesis. The 'oxidation theory' of atherogenesis envisages (per)oxidation of LDL as a crucial event resulting in formation of foam cells and triggering of various pathways leading to development of the disease (1–3). Although the oxidant(s) responsible for in vivo oxidation of LDL are unknown at present, a range of radical and nucleophilic oxidants, including 15-lipoxygenase (4), myeloperoxidase (5, 6), endothelial nitric oxide synthase (7), or transition metals, either free or protein-bound (8–10) have been implicated.

Indirect evidence implicating oxidation of lipoproteins in atherogenesis comes from studies showing that different antioxidants inhibit LDL oxidation in vitro (11), and that supplementation with some (12–14), though not all (15) antioxidants, retards lesion forma-

Abbreviations: AAPH, 2,2′-azo*bis*(2-amidinopropane) hydrochloride; apoE, apolipoprotein E; apoE$^{-/-}$, apoE knockout; CE-OH, cholesteryl ester hydroxides; CE-OOH, cholesteryl ester hydroperoxides; CE-O(O)H, cholesteryl ester hydroxides plus hydroperoxides; Ch18:1, cholesteryl oleate; Ch18:2, cholesteryl linoleate; Ch20:4, cholesteryl arachidonate; FC, free cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; LPDP, lipoprotein-deficient plasma; v, lipid peroxidation chain length; PBS, phosphate-buffered saline; PC-OOH, phosphatidyl choline hydroperoxides; ROO$^•$, aqueous peroxyl radicals derived from AAPH: R_g, rate of radical generation; R_i, rate of lipid peroxidation initiation; R_p, rate of lipid peroxidation; TMP, tocopherol-mediated peroxidation; α-TO$^•$, α-tocopheroxyl radical; α-TOH, α-tocopherol; VLDL, very low density lipoprotein; β-VLDL, β-migrating very low density lipoprotein.

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tion in animal models of atherosclerosis. The reasons for these conflicting results are presently unknown. They may include differences in either the underlying causes of atherogenesis or the mechanism(s) of lipoprotein oxidation in the different animals used in these models. Regarding the latter, most mechanistic studies addressing lipoprotein oxidation and antioxidation have used human LDL and focused on the lipid moieties, as lipid peroxidation is thought to precede and contribute to the oxidative modifications of apolipoprotein B-100 (16).

We have proposed and experimentally verified tocopherol-mediated peroxidation (TMP) as a model of human lipoprotein lipid peroxidation. TMP departs from the classical model of lipid peroxidation, where α-tocopherol (α-TOH) (by far the most abundant antioxidant in extracts of LDL) acts as a chain-breaking antioxidant. The classic model, in contrast to TMP, is unable to explain several features of radical-induced LDL lipid peroxidation and its inhibition by antioxidants. In TMP, α-tocopheroxyl radical (α-TO$^\bullet$) is formed either directly by the interaction of α-TOH on the surface of LDL with the initiating (radical) oxidant, or indirectly after initial formation of a lipid peroxyl radical. In any case, the resulting α-TO$^\bullet$ can then propagate lipid peroxidation within the isolated lipoprotein particle (17).

This pro-oxidant effect of α-TOH is affected by several conditions. These include the type of radical oxidant involved and the frequency with which LDL encounters this oxidant. We and others have recently shown that by varying these conditions, α-TOH can switch from a pro-oxidant into an antioxidant (18, 19), and that the pro-oxidant effect of α-TOH can be observed not only in isolated lipoproteins but also in human plasma (18, 20). Another important feature that prevents the pro-oxidant activity of α-TOH and makes the vitamin an antioxidant is the presence of co-antioxidants, i.e., compounds that are capable of eliminating the (single) radical from an oxidizing lipoprotein particle via initial reduction of α-TO$^\bullet$ (21). The fact that α-TOH is such an avid radical scavenger, and that the α-TO$^\bullet$ is a poor oxidant yet rapidly interacts with co-antioxidants, makes vitamin E an excellent antioxidant in situations where co-antioxidants are not deficient (21). We have identified several co-antioxidants (22, 23), some of which are present in the circulation (24), interstitial fluids (25), or in homogenates of advanced human atherosclerotic plaque (26).

In the search for animal models of atherogenesis, apolipoprotein E gene knockout (apoE$^{-/-}$) mice were generated recently by introducing a missense mutation in the apoE gene by homologous recombination in embryonic stem cells (27). ApoE serves as a ligand for receptors for lipoproteins and hence plays a crucial role in lipoprotein clearance. Indeed, apoE$^{-/-}$ mice have a different metabolism of circulating lipoproteins, compared to the normolipidemic background animals: they accumulate large amounts of chylomicron and VLDL remnants, pro-atherogenic lipoproteins containing low amounts of triglycerides, and large amounts of cholesteryl esters. ApoE$^{-/-}$ mice spontaneously develop arterial lesions (27–29), and this process is enhanced by a high fat, Western diet. Recent reports suggest that atherogenesis in the apoE$^{-/-}$ mouse involves disregulation of the normal immune response (30, 31) as well as the oxidative modification of lipoproteins (32). Evidence for the latter includes increased titers of circulating autoantibodies to oxidized lipoproteins, and the presence in atherosclerotic lesions of epitopes recognized by antibodies against oxidized LDL (33). Perhaps the strongest evidence for a role of lipoprotein oxidation in atherogenesis in apoE$^{-/-}$ mice comes from a study showing that dietary supplementation with the antioxidant *N,N′*-diphenyl 1,4-phenylenediamine (an efficient anti-TMP agent (23)), resulted in a significant reduction of atherosclerosis and increased resistance of lipoproteins isolated from plasma of such animals to Cu(II)-induced oxidation (34).

To examine a potential role of TMP in atherogenesis, we oxidized plasma and isolated lipoproteins from apoE$^{-/-}$ mice and the normolipidemic, background C57BL/6J and normal CBA mice, using aqueous peroxyl radicals (ROO$^\bullet$) generated at constant rates by the thermolabile azo-initiator 2,2′-azo*bis*(2-amidinopropane) hydrochloride (AAPH). We report here that circulating lipids from the apoE$^{-/-}$ mice are susceptible to peroxidation whereas those of the background and control mice are surprisingly oxidant resistant.

MATERIAL AND METHODS

Animals

CBA mice (males, 6–8 weeks old) were from the Department of Pathology, University of Sydney, and the C57BL/6J mice (males, 5–8 weeks old) were either provided by the Biological Facility of the Heart Research Institute or purchased from the Animal Resources Center (Perth, Australia), which also supplied homozygous apoE$^{-/-}$ mice (males, 5–8 weeks old) derived from a colony of the original breeders (27). These animals were maintained in a virus-free environment at the Heart Research Institute Biological Facility and fed a standard chow (Lab-Feed, Sydney, Australia) ad libitum.

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Preparation of plasma and lipoproteins

Blood was drawn from anesthetized mice by cardiac puncture using 1-ml tuberculin syringes containing ≈50 μl 0.1% heparin. Blood obtained from healthy normolipidemic human subjects was drawn into heparinized Vacutainers (Becton Dickinson, UK). Immediately after bleeding, blood cells were separated from plasma (2,000 *g*, 20 min, 4°C), of which an aliquot was taken for initial lipid and antioxidant determination (see below). Plasma was used either for oxidation experiments or lipoprotein preparation. The latter was achieved by density ultracentrifugation (417,000 *g*, 3 h, 15°C) using the TL-100 bench-top ultracentrifuge fitted with a TLA-100.4 or TLA-100.2 rotor (Beckman, Palo Alto, CA) (35). As mouse plasma contains little carotenoids, the lipoprotein bands are difficult to localize after ultracentrifugation. We therefore estimated their positions by running, in parallel, tubes containing normal human plasma or control mouse plasma to which Coomassie Brilliant Blue R-250 (Sigma, ≈30 μl 5%, w/v, in phosphate-buffered saline (PBS), per 1 ml density-adjusted plasma) was added prior to ultracentrifugation. To avoid contamination with material from neighboring bands, the central portion of individual lipoprotein 'bands' only was removed by aspiration.

Isolation of 'VLDL' from human atherosclerotic plaque

Human lesion 'VLDL' was prepared from carotid plaque obtained within 15–60 min of surgical removal from patients undergoing carotid endarterectomy. Plaques were rinsed immediately in Chelex®-100-treated and argon-flushed PBS containing butylated hydroxytoluene (10 μmol/l), and processed immediately as follows. After dissecting away the surrounding adventitia and media, plaques were rinsed in PBS, blotted dry with tissue, weighed, and added to Chelex-treated and argon-flushed sodium carbonate (100 mmol/l)/EDTA (2.7 mmol/l) buffer (pH 11), containing butylated hydroxytoluene (10 μmol/l), gentamycin (0.008% v/v), chloramphenicol (0.008% w/v), and a cocktail of protease inhibitors (Protease Inhibitor Cocktail Tablet, Boehringer Mannheim). Plaques (147 mg wet weight/ml buffer) were then minced and homogenized at 4°C for 5 min as described (26). The homogenate was centrifuged (2,000 *g*, 4°C, 10 min) and 2 ml of the supernatant (adjusted to density of 1.21 g/ml with solid KBr) underlayered in a 5.1-ml centrifuge tube (Beckman) containing 3.1 ml of Chelex-treated PBS, and subjected to density gradient ultracentrifugation (417,000 *g*, 15°C, 4 h) using the TL-100 ultracentrifuge equipped with the TLA-100.4 rotor. After ultracentrifugation, the tube was pierced with a needle and the top 700 μl of its content (density < 1.023 g/ml; referred to as lesion 'VLDL') removed with a syringe.

Lesion 'VLDL' were positive for apolipoprotein B-100 (SDS-PAGE/Western blot) and contained (mean ± SD, n = 5) 49.6 ± 8.5 μg total protein/ml, 3.95 ± 1.2 nmol free cholesterol (FC)/μg protein, 0.8 ± 0.23 mol cholesteryl linoleate (Ch18:2) per mol FC, 0.15 ± 0.02 mol cholesteryl arachidonate (Ch20:4) per mol FC, and 18.9 ± 7.3 mmol α-TOH/mol Ch18:2.

Oxidation of plasma and lipoproteins

Oxidations were carried out under air and at 37°C for the period of time indicated. AAPH (10 mmol/l for plasma and 1 mmol/l for isolated lipoproteins, unless stated otherwise) was used to initiate oxidation. AAPH is a thermolabile radical initiator that decomposes to yield two ROO$^\cdot$ per molecule of AAPH at a constant rate ($R_g{}^{37°C}$, = 1.3×10^{-6} [*AAPH*]/s), where [*AAPH*] is the concentration of the initiator in mmol/l (36, 37)). Where indicated, plasma was supplemented with α-TOH dissolved in DMSO prior to the addition of AAPH. Oxidations were terminated by extracting an aliquot of the reaction mixture using an ice-cold biphasic extraction system (see below). For some oxidation experiments, the lipid peroxidation chain length (v) was calculated according to $v = R_p/R_i$, where $R_p = d[CE\text{-}O(O)H]/dt$ is the cholesteryl ester peroxidation rate, and $R_i = -2\ d[\alpha\text{-}TOH]/dt$ is the peroxidation initiation rate (17). R_p refers to the rate at which hydroperoxides (CE-OOH) and the corresponding hydroxides (CE-OH) of Ch18:2 and Ch20:4 (together referred to as CE-O(O)H) accumulated during the period of maximum rate of α-TOH consumption (see (17) for detail). In experiments where the effect of different R_p on v was tested, AAPH was used at 10 and 2 mmol/l (plasma), or at 1 and 0.2 mmol/l (VLDL of apo$E^{-/-}$ mouse plasma).

To investigate the effect of plasma proteins on the oxidation of human and apo$E^{-/-}$ mouse lipoproteins, mouse and human lipoprotein-deficient plasma (LPDP) was obtained by aspiration of the most dense fraction (d > 1.21 g/ml) obtained after density ultracentrifugation of the corresponding plasma samples. Total lipoproteins were obtained by combining high-density lipoproteins (HDL) with all other, more buoyant lipoproteins, followed by concentration (≈2-fold) in a Centricon-30 unit (Amicon), and gel filtration through a PD-10 column to remove low-molecular weight components (Pharmacia). LPDP and total lipoproteins were then reconstituted as indicated in the corresponding figure, supplemented with 5 mmol/l AAPH, incubated at 37°C, and aliquots taken and analyzed for α-TOH and CE-O(O)H.

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The stability of CE-OOH in mouse and human plasma was studied by addition of human LDL (≈1 μmol/l), pre-oxidized with AAPH (incubation of ≈1 μmol/l LDL with 1 mmol/l AAPH at 37°C for 1 h followed by removal of AAPH by gel filtration), to apoE$^{-/-}$ mouse or human plasma and incubation at 37°C. At various times, aliquots were extracted and analyzed for CE-O(O)H.

Size-exclusion chromatography of plasma

Freshly prepared samples of plasma obtained from C57BL/6J and apoE$^{-/-}$mice were subjected to size-exclusion chromatography using the Fast Protein Liquid Chromatography (FPLC™) system (Pharmacia) fitted with a Superose-6 (Pharmacia) column (30 × 1.5 cm i.d.). Plasma samples (1 ml ≈ 30 mg protein) were loaded onto the column equilibrated and eluted with 20 mm sodium phosphate buffer (pH 7.8) at a flow rate of 0.25 ml/min. The eluant was monitored at 279 nm and 0.5-ml fractions were collected for lipid and protein analyses (see below).

Analysis of antioxidants, lipids, and lipid oxidation products

Aliquots (50–400 μl) of the various samples were extracted into 1 ml of cold methanol (containing 0.1% (v/v) acetic acid) and 5 ml of hexane, vortexed vigorously for 30 s, and centrifuged at 4°C and 1,000 *g* for 5 min. Four ml of the resulting hexane layer was removed, dried in vacuo, and the residue was resuspended in 200 μl isopropanol and used for subsequent HPLC analysis of the lipophilic antioxidants, α-tocopherylquinone, FC, cholesteryl esters and CE-O(O)H (see below). The aqueous methanol phase was removed, filtered (0.2 μm), and used for analysis of hydroperoxides of phosphatidylcholine (PC-OOH) by HPLC post-column chemiluminescence detection (35). For analysis of ascorbate and urate, an aliquot of the filtered aqueous methanol phase was kept on dry ice until analyzed (<4 h). The lipophilic antioxidants and unoxidized FC and cholesteryl esters present in the hexane extract were analyzed using reversed-phase HPLC with electrochemical and UV_{210} detection, respectively, as described (35). For oxidized lipids, the reversed-phase HPLC was combined with either post-column chemiluminescence (for specific and sensitive monitoring of CE-OOH), or UV_{234} detection (for CE-O(O)H). In some cases, these extracts were subjected to an RP-HPLC method separating the cholesteryl linoleate hydroperoxide from cholesteryl linoleate hydroxide (38). In other cases, the extracts were also used for analysis of α-TOH, α-tocopheryl quinone, and α-tocopheryl hydroquinone by HPLC with electrochemical detection (39). Ascorbate and urate were analyzed by HPLC with electrochemical detection (40). The individual components were quantified by area comparison with corresponding authentic standards. PC-OOH and CE-OOH standards were prepared by oxidation of soybean phosphatidyl choline (Sigma) and Ch18:2 (Sigma), respectively, using the lipophilic azo-initiator 2,2′-azo*bis*(2,4-dimethylvaleronitrile) (Polysciences) (35). Authentic standard of hydroxy 13-(S) (*Z,E*) Ch18:2 was purchased from Cayman Chemicals (Ann Arbor, MI). Cholesteryl benzoate and isoascorbate (both Sigma) were used as external standards for the analysis of the lipophilic and aqueous plasma components, respectively. For this, cholesteryl benzoate (200 nmol) and isoascorbate (500 pmol) were added to plasma aliquots prior to extraction. Endogenous free cholesterol was used as internal standard for the analysis of lipophilic compounds in the lipoprotein extracts.

Other methods

Protein concentration in the plasma and lipoprotein samples was determined using the bicinchoninic acid assay kit (Sigma) with bovine serum albumin as the standard. HDL was determined by the dextrane sulfate precipitation method (41). SDS-PAGE was performed using a Bio-Rad MiniProtean II system and pre-cast 4–15% gels (Bio-Rad), with protein bands visualized by silver staining. Western blotting was carried out after transfer of the separated proteins onto a nylon membrane, and using a monoclonal mouse anti-apolipoprotein E (Biodesign International, Kennebunk, ME) and human anti-apolipoprotein B-100 antibody (Serotec, UK) and the ECL visualization system.

RESULTS

Exposure of blood plasma obtained from the C57BL/6J mice to a constant flux of ROO$^{\bullet}$ generated from AAPH resulted in a rapid and linear consumption of ascorbate (**Fig. 1A**). After depletion of endogenous ascorbate, the levels of plasma α-TOH and urate decreased in a time-dependent manner, so that after 7 h of incubation none of the major non-proteinaceous antioxidants remained present. These results are similar, though not entirely identical, to those obtained with human plasma undergoing AAPH-induced oxidation, where ascorbate consumption is followed by that of bilirubin, urate, and α-TOH (24). Mouse plasma contains comparatively much smaller amounts of bilirubin (42), the consumption of which we did not follow here. Exposure of plasma from C57BL/6J mice to AAPH did not result in the accumulation of substantial amounts of CE-O(O)H, even for periods of time be-

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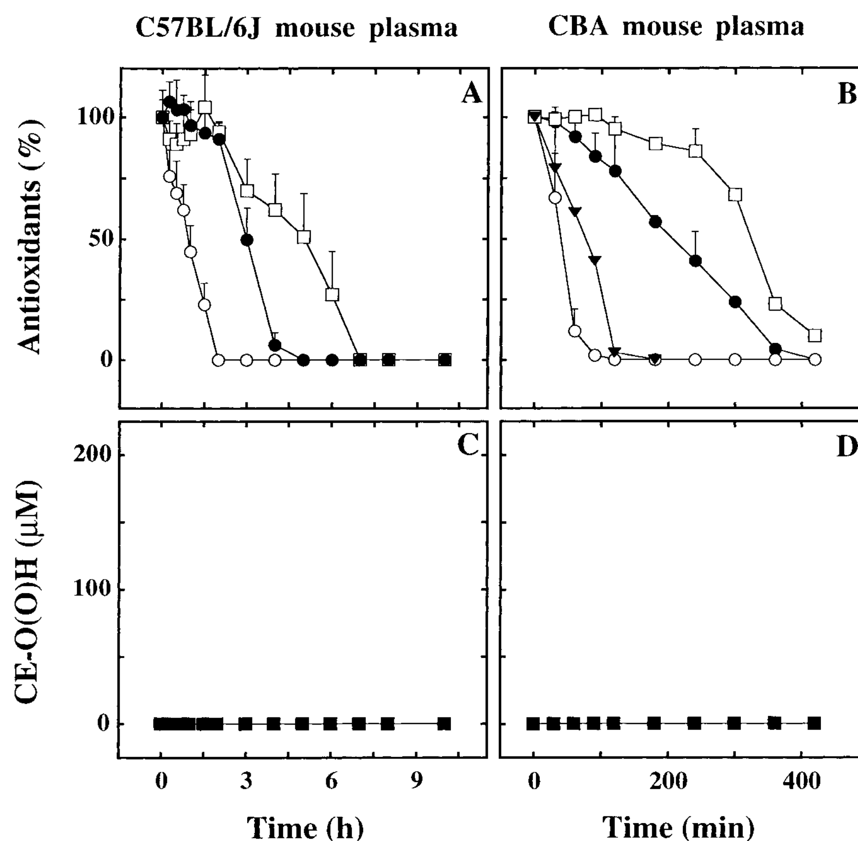

Fig. 1. Plasma of the normolipidemic C57BL/6J and CBA mice is resistant to lipid oxidation. Plasma prepared from blood of C57BL/6J (A, C) and CBA mouse (B, D) was supplemented with 10 mmol/l AAPH and incubated at 37°C. At the time points indicated, plasma aliquots were taken, extracted, and analyzed for the antioxidants (A, B) ascorbate (○), ubiquinol-9 (▼), α-TOH (●) and urate (□), and for CE-O(O)H (C, D; ■) as described in Materials and Methods. The initial antioxidant concentrations (in μmol/l) were 19 ± 2.1, 35.1 ± 4.5, and 98.9 ± 21.5 for α-TOH, ascorbate, and urate, respectively, in C57BL/6J mouse plasma, and 24.9 ± 1.3, 30.3 ± 2.9, and 130.9 ± 5.6 for α-TOH, ascorbate, and urate, respectively, in CBA mouse plasma. Data shown are mean values ± SD of three separate experiments.

yond complete consumption of ascorbate and the other antioxidants (Fig. 1C). This is in sharp contrast to the situation in human plasma, where under comparable oxidizing conditions CE-O(O)H accumulate after depletion of ascorbate and concomitantly with α-TOH consumption (24). The formation of very low levels of CE-O(O)H during AAPH-induced oxidation appeared to be a general feature of mouse plasma rather than a peculiarity of the C57BL/6J mice, as exposure of plasma obtained from the CBA mice to this oxidant (Fig. 1D) also resulted in CE-O(O)H levels well below those observed with human plasma.

The absence of substantial amounts of detectable CE-O(O)H in mouse plasma undergoing AAPH-induced oxidation was conceivably due to the presence of a reducing activity (43) converting CE-OOH into the corresponding CE-OH. The latter cannot be detected by the HPLC post-column chemiluminescence method used. However, using HPLC with UV_{234} detection also failed to detect substantial amounts of CE-OH and CE-OOH in mouse plasma undergoing AAPH-induced oxidation (Fig. 1), even when a different reversed-phase HPLC method (38) was used that fully separated Ch18:2-OH from Ch18:2-OOH (data not shown).

Mouse plasma differs from human plasma in that the former contains HDL as the predominant lipoprotein and almost exclusive carrier of circulating α-TOH. Determining the concentration of HDL and α-TOH in plasma of CBA mice, and assuming uniform distribution of the vitamin among different HDL particles, showed that only one in nine HDL particles contained, on average, one molecule of α-TOH (data not shown). This, together with our previous observation that cholesteryl esters in isolated lipoproteins and in plasma depleted of their α-TOH are largely resistant to oxidation initiated by a low flux of $ROO^•$ (18), suggested that the

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absence of α-TOH from most HDL particles contributed to the observed lack of substantial lipid peroxidation in mouse plasma exposed to AAPH. To test this directly, we compared the oxidizability of plasma from CBA mice before and after addition of α-TOH. **Figure 2** shows that significantly more CE-O(O)H accumulated in normolipidemic mouse plasma to which α-TOH was added when compared with its unsupplemented, native counterpart. Further, accumulation of CE-OOH occurred while α-TOH was still present, though only after depletion of endogenous ascorbate (not shown).

We next tested the oxidizability of plasma obtained from the apoE$^{-/-}$ mice towards peroxyl radicals derived from AAPH (10 mmol/l), and compared the results with those obtained with plasma from the background C57BL/6J mice (cf. Fig. 1A, C). Ascorbate was the first antioxidant to be depleted, at a rate similar to that in plasma prepared from the C57BL/6J mice, and CE-O(O)H was not detected as long as ascorbate was present (**Fig. 3**). After consumption of ascorbate, urate

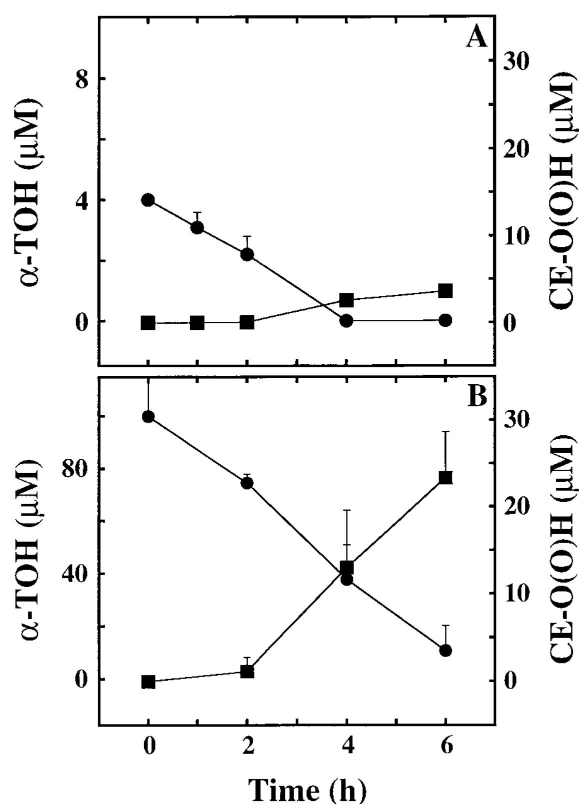

Fig. 2. Supplementation of CBA mouse plasma with α-TOH increases its oxidizability. Control (A) or α-TOH-supplemented (100 μmol/l) plasma (B) prepared from the CBA mouse was incubated at 37°C in the presence of 10 mmol/l AAPH. At the time points indicated, aliquot was taken and analyzed for α-TOH (●) and CE-OOH (■) as described in Materials and Methods. Data shown are mean values of three separate experiments ± SD.

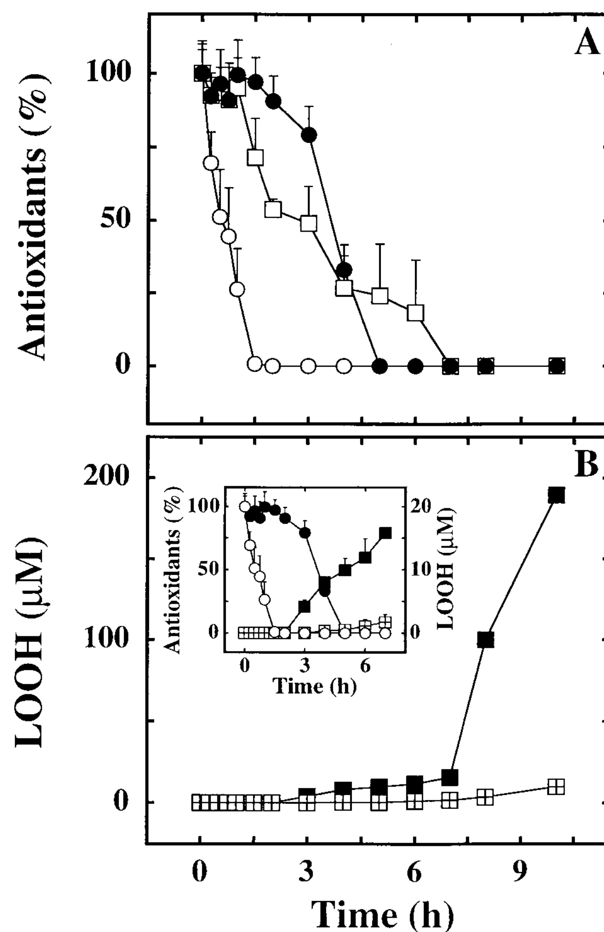

Fig. 3. Plasma of the hyperlipidemic apoE$^{-/-}$ mice is readily oxidizable. Plasma was prepared from apoE$^{-/-}$ mouse, supplemented with 10 mmol/l AAPH, and incubated at 37°C. At the time points indicated, aliquots were withdrawn, extracted, and analyzed for antioxidants (A) (ascorbate, ○; α-TOH, ●; urate, □) and oxidized lipids (B) (PC-OOH, ⊞; CE-O(O)H, ■) as described in Materials and Methods. The inset in panel B shows the data points from panel B for the first 7 h of oxidation. The initial plasma values were 49.1 ± 5.8, 35.9 ± 4.8, and 101 ± 12.9 μmol/l for α-TOH, ascorbate, and urate, respectively. The data shown are mean values ± SD from 3 independent experiments.

and α-TOH disappeared at rates somewhat different from those observed in plasma of the C57BL/6J mice. This may reflect differences in the plasma α-TOH content between the two strains of mice (see below). More strikingly, however, AAPH-induced oxidation of plasma from the apoE$^{-/-}$ mice caused substantial accumulation of lipid hydroperoxides, such that about 180 μmol/l CE-O(O)H and 15 μmol/l PC-OOH accumulated in the first 10 h of oxidation, while within the same period of incubation very little CE-O(O)H was detected in the C57BL/6J mouse plasma (cf. Fig. 1C). The onset of lipid hydroperoxide accumulation in

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plasma from the apoE$^{-/-}$ mice coincided with the onset of α-TOH consumption (Fig. 3, inset).

In spite of the high level of lipid peroxidation in apoE$^{-/-}$ mouse plasma compared to that of C57BL/6J mice, calculation of v (see Methods) revealed that it was <1 during the period of α-TOH consumption. This is in contrast to corresponding studies of AAPH-induced oxidation of human plasma where $v \approx 10–20$ was obtained (24). TMP predicts (17) that for a given lipoprotein concentration, v is inversely proportional to R_g. To test this prediction for mouse plasma lipid peroxidation, we exposed apoE$^{-/-}$ mouse plasma to 10 and 2 mmol/l AAPH. As expected, this resulted in different R_i, as assessed by the different rates of α-TOH consumption after all ascorbate was oxidized (**Fig. 4**). In parallel, R_p varied proportionally, so that v was close to 1 in both cases. These results suggest that (apoE$^{-/-}$) mouse plasma contains factor(s) in addition to ascorbate that prevent(s) v reaching values >1.

To rule out degradation of the initially formed CE-OOH in AAPH-initiated oxidation of mouse plasma as a possible reason for the relatively low rate of CE-O(O)H accumulation, we tested the stability of CE-OOH in mouse and human plasma. Thus, when pre-oxidized human LDL (≈13.7 μmol/l CE-OOH final concentration) was added to plasma of apoE$^{-/-}$ mice and humans, the added CE-OOH was converted quantitatively to CE-OH at 1.9 and 1.5 μmol/l per h, respectively. This indicated that a loss of CE-OOH for reasons other than their reduction to CE-OH (see above) is unlikely to explain the relatively lower levels of CE-O(O)H accumulating in AAPH-induced oxidation of mouse vs. human plasma.

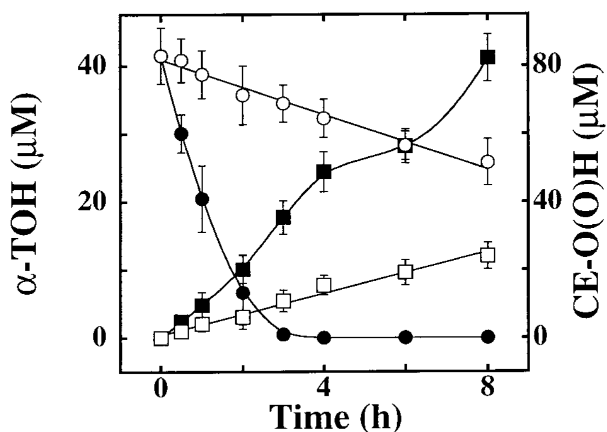

Fig. 4. The radical chain length in apoE$^{-/-}$ mouse plasma exposed to AAPH is not dependent on the rate of radical generation. Plasma from apoE$^{-/-}$ mice was supplemented with 2 (open symbols) or 10 mm (closed symbols) AAPH and incubated at 37°C. At the time points indicated, aliquots were withdrawn, extracted, and analyzed for α-TOH (circles) and CE-O(O)H (squares) as described in Materials and Methods. The data shown are mean values ± SD of three experiments.

Table 1 compares the levels of protein, free cholesterol, cholesteryl esters and antioxidants in plasma of C57BL/6J and apoE$^{-/-}$ mice. As can be seen, there was no significant difference in the concentrations of protein, aqueous antioxidants, and Ch20:4, while the contents of FC, Ch18:2, and cholesteryl oleate (Ch18:1) were 4-, 3-, and 12-fold higher, respectively, in plasma from the apoE$^{-/-}$ mice. This is consistent with previous reports demonstrating accumulation of cholesteryl ester-enriched β-VLDL particles in plasma of the apoE$^{-/-}$ mice (28, 44). In parallel with the ≈3-fold increase in Ch18:2, quantitatively the major single oxidizable lipid in human LDL (11), plasma levels of α-TOH were also increased ≈3-fold in the apoE$^{-/-}$ mouse plasma (Table 1), resulting in comparable plasma α-TOH-to-Ch18:2 ratios for the C57BL/6J and apoE$^{-/-}$ mice.

It has been shown previously that plasma from apoE$^{-/-}$ mice contains increased levels of larger, more buoyant lipoproteins at the expense of dense lipoproteins when compared to the C57BL/6J mouse plasma (27). The results shown in **Fig. 5** and **Table 2** are consistent with this and show, for the first time to our knowledge, the plasma distribution of α-TOH, Ch18:2, and Ch20:4 for apoE$^{-/-}$ and C57BL/6J mice. In plasma of normolipidemic C57BL/6J mice most lipid was associated with lipoproteins (referred to as M-HDL) of the size range of human HDL, whereas lipids in the plasma of apoE$^{-/-}$ mice were largely associated with lipoproteins of the size of human very low density lipoprotein (M-VLDL, also referred to as β-VLDL) and, to some extent, LDL (M-LDL) (Fig. 5). For both strains of mice, the distribution of circulating α-TOH 'mirrored' that of Ch18:2 and Ch20:4 (Fig. 5). Thus in plasma of the normolipidemic C57BL/6J mice almost 90% of α-TOH was present in the M-HDL fraction (Table 2), whereas in apoE$^{-/-}$ mice 57 and 37% of the vitamin were associ-

TABLE 1. C57BL/6J and apoE$^{-/-}$ mouse plasma lipids and antioxidants

Component	n	C57BL/6J	ApoE$^{-/-}$
Protein, mg/ml	5	35.8 ± 4.7	34.8 ± 6.4
FC, mmol/l	4	0.93 ± 0.08	3.74 ± 0.28
Ch20:4, mmol/l	4	0.8 ± 0.19	1.02 ± 0.1
Ch18:2, mmol/l	4	1.19 ± 0.17	3.48 ± 0.14
Ch18:1, mmol/l	4	0.32 ± 0.04	4.1 ± 0.6
α-TOH, μmol/l	4	18.5 ± 1.7	51.3 ± 5
α-TOH/LH[a]		2.77 ± 0.25	3.13 ± 0.31
Ascorbate, μmol/l	4	35.3 ± 3.3	36.4 ± 4.5
Urate, μmol/l	4	99.4 ± 17.5	95.6 ± 11.1

[a]α-TOH per bisallylic hydrogen refers to the ratio of α-TOH to Ch18:2 plus Ch20:4.

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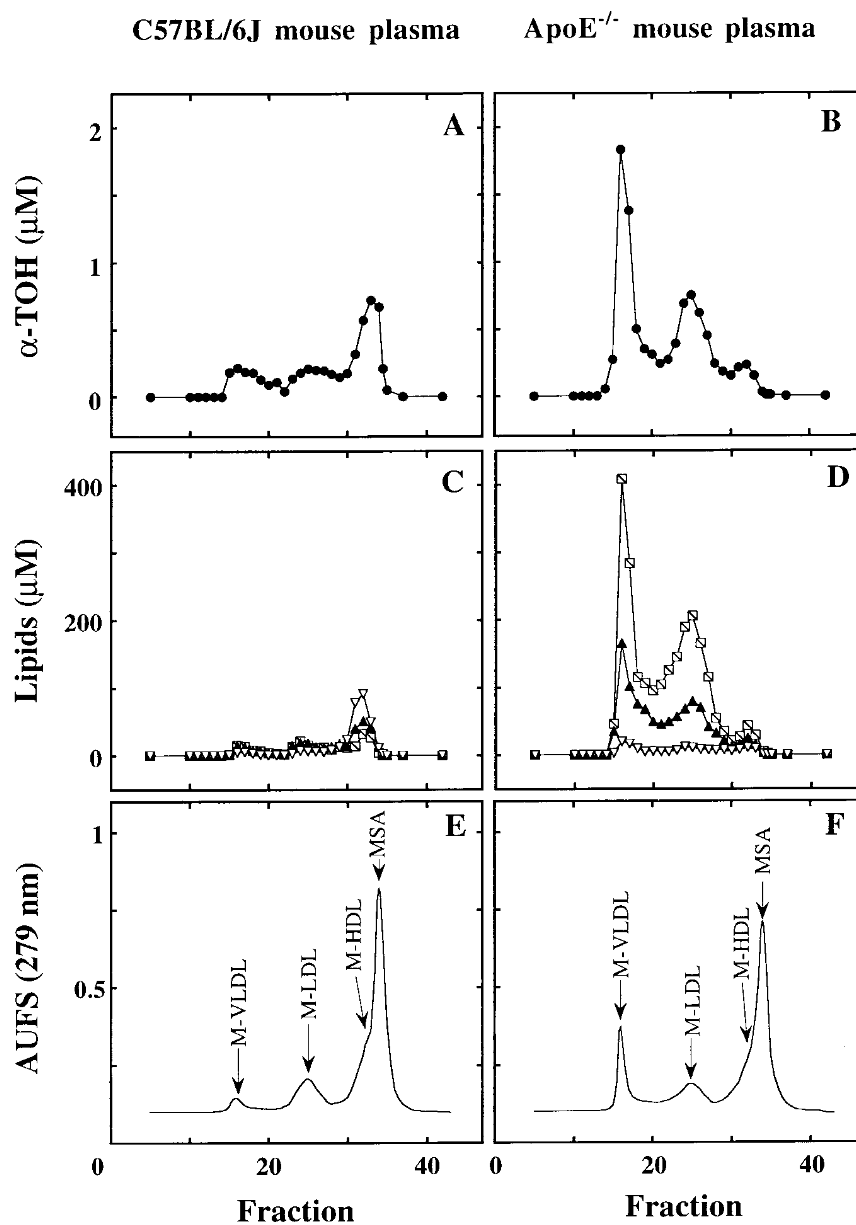

Fig. 5. The distribution of α-TOH and lipids in circulating lipoproteins is different in C57BL/6J and apoE$^{-/-}$ mice. Plasma from C57BL/6J (panels A, C, E) and apoE$^{-/-}$ mice (panels B, D, F) was fractionated by FPLC using a Superose-6 column (Pharmacia) eluted with 0.2 mm phosphate buffer (pH 7.4) at 0.25 ml/min, and the eluant was monitored at 279 nm (E, F). The positions of M-VLDL, M-LDL, M-HDL, and mouse serum albumin (MSA) in these panels are indicated by arrows above the corresponding peaks. Each fraction was extracted and analyzed for α-TOH (A, B), and FC (⧅), Ch20:4 (▽), and Ch18:2 (▲) (C, D). The profiles shown are derived from one experiment using plasma pooled from several mice and are, both qualitatively and quantitatively, very similar to those obtained in two other independent experiments.

ated with the more buoyant M-VLDL and M-LDL fractions, respectively (Table 2).

Figure 6 illustrates that, in addition to the differences in α-TOH content, there was also a marked difference in the lipid profile between M-VLDL preparations from the C57BL/6J and apoE$^{-/-}$ mice plasma. Thus, the former was relatively rich in triglycerides and poor in FC and cholesteryl esters, whereas the converse was true for M-VLDL from the apoE$^{-/-}$ mouse plasma. As apoE$^{-/-}$ mice have been proposed as an animal model

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TABLE 2. C57BL/6J and apoE$^{-/-}$ lipoprotein lipid values

	C57BL/6J Mouse			ApoE$^{-/-}$ Mouse		
	M-VLDL[a]	M-LDL	M-HDL	M-VLDL	M-LDL	M-HDL
FC[b]	0.04	0.24	0.71	0.7	0.2	0.1
Ch20:4	0.01	0.13	0.86	0.74	0.16	0.08
Ch18:2	0.01	0.18	0.81	0.78	0.16	0.06
Ch18:1	0.12	0.44	0.44	0.81	0.17	0.02
α-TOH	0.02	0.1	0.88	0.53	0.37	0.1

[a]M-VLDL, M-LDL, and M-HDL lipoprotein fractions correspond to fractions 10–15, 20–25, and 30–35, respectively, in plasma FPLC fractionation (cf. Fig. 5).

[b]Fractional concentration of FC, Ch20:4, Ch 18:2, Ch18:1, and α-TOH in individual lipoproteins was calculated as relative molar concentration in the lipoprotein fractions (see above) where $F_{M\text{-}VLDL} + F_{M\text{-}LDL} + F_{M\text{-}HDL} = 1$.

The results shown are mean values from three independent experiments with error ≤10%.

to study the oxidation theory of atherogenesis (45), we also compared the lipid profile of M-VLDL, the major lipoprotein in the apoE$^{-/-}$ mouse plasma (Fig. 5), with that of the 'VLDL' fraction isolated from an advanced human atherosclerotic lesion. As can be seen, the two lipid profiles were very similar, at least with regard to the relative concentrations of α-TOH, FC, triglycerides, and oxidizable cholesteryl esters (Fig. 6, middle and bottom panel, respectively).

Next, we subjected the individual lipoprotein fractions isolated from pooled plasma samples of C57BL/6J and apoE$^{-/-}$ mice to mild peroxyl radical-induced oxidation (i.e., conditions where TMP is operative for human lipoproteins (17)), and monitored the accumulation of CE-O(O)H during the early period of oxidation when α-TOH is present. For this we used the isolated lipoproteins at concentrations that reflected the situation in the respective plasma samples. M-HDL isolated from plasma of C57BL/6J mice (**Fig. 7E**) provided most of the relatively small amounts of detectable CE-O(O)H, whereas for apoE$^{-/-}$ mice this lipoprotein fraction yielded least oxidized lipids (Fig. 7F). Conversely, very little to no CE-O(O)H accumulated in the α-TOH-poor M-LDL and M-VLDL fractions isolated from C57BL/6J mice (Figs. 7A, C), whereas for apoE$^{-/-}$ mice, these two α-TOH-rich lipoprotein fractions yielded large amounts of oxidized lipids (Figs. 7B, D). Also, *v* for the peroxidation of cholesteryl esters (see Methods) in M-VLDL from apoE$^{-/-}$ mouse plasma during the α-TOH-containing period was ≈3.5, which is within the range found previously for comparable oxidation experiments using isolated human LDL (46). Overall, these results demonstrate that despite the marked differences between the extent and origin of accumulating CE-O(O)H in lipoproteins from C57BL/6J and apoE$^{-/-}$ mouse plasma, there was a direct correlation between the α-TOH content and amounts of

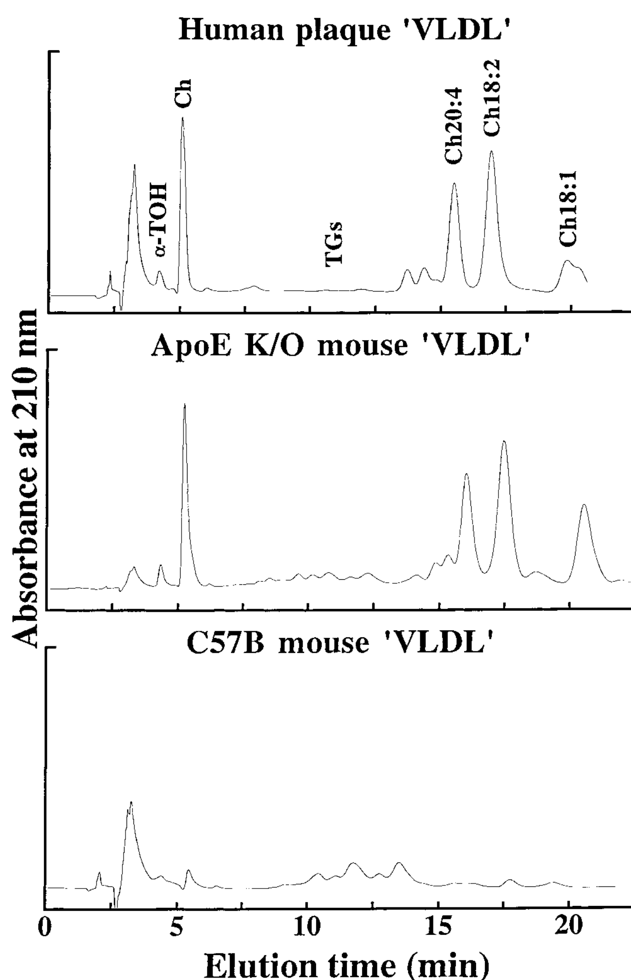

Fig. 6. The lipid composition of M-VLDL isolated from apoE$^{-/-}$ mouse plasma differs from that of C57BL/6J mouse plasma, but resembles that of VLDL isolated from advanced human atherosclerotic plaques. Lipoproteins, isolated from the C57BL/6J (bottom panel) and apoE$^{-/-}$ mouse plasma (middle panel), and from human atherosclerotic plaque (upper panel) as described in Materials and Methods, were extracted and the organic phase analyzed for lipophilic components by HPLC with UV_{210nm} absorbance. The traces, showing the positions of α-TOH, FC, triglycerides (TGs), Ch20:4, Ch18:2, and cholesteryl oleate (Ch18:1), are qualitatively and quantitatively similar to chromatograms obtained from >10 different M-VLDL samples of C57BL/6J and apoE$^{-/-}$ mice and VLDL fractions prepared from 6 different human atherosclerotic plaques.

oxidized lipids formed in the different lipoproteins. Peroxyl radical-induced oxidation of identical concentrations (based on their content of Ch18:2) of either M-LDL and M-HDL from the two mouse strains resulted in comparable rates of α-TOH depletion and CE-O(O)H accumulation (data not shown), indicating that there was no difference in the oxidizability of the isolated lipoproteins from the two mouse strains. C57BL/6J mice did not contain sufficient M-VLDL to

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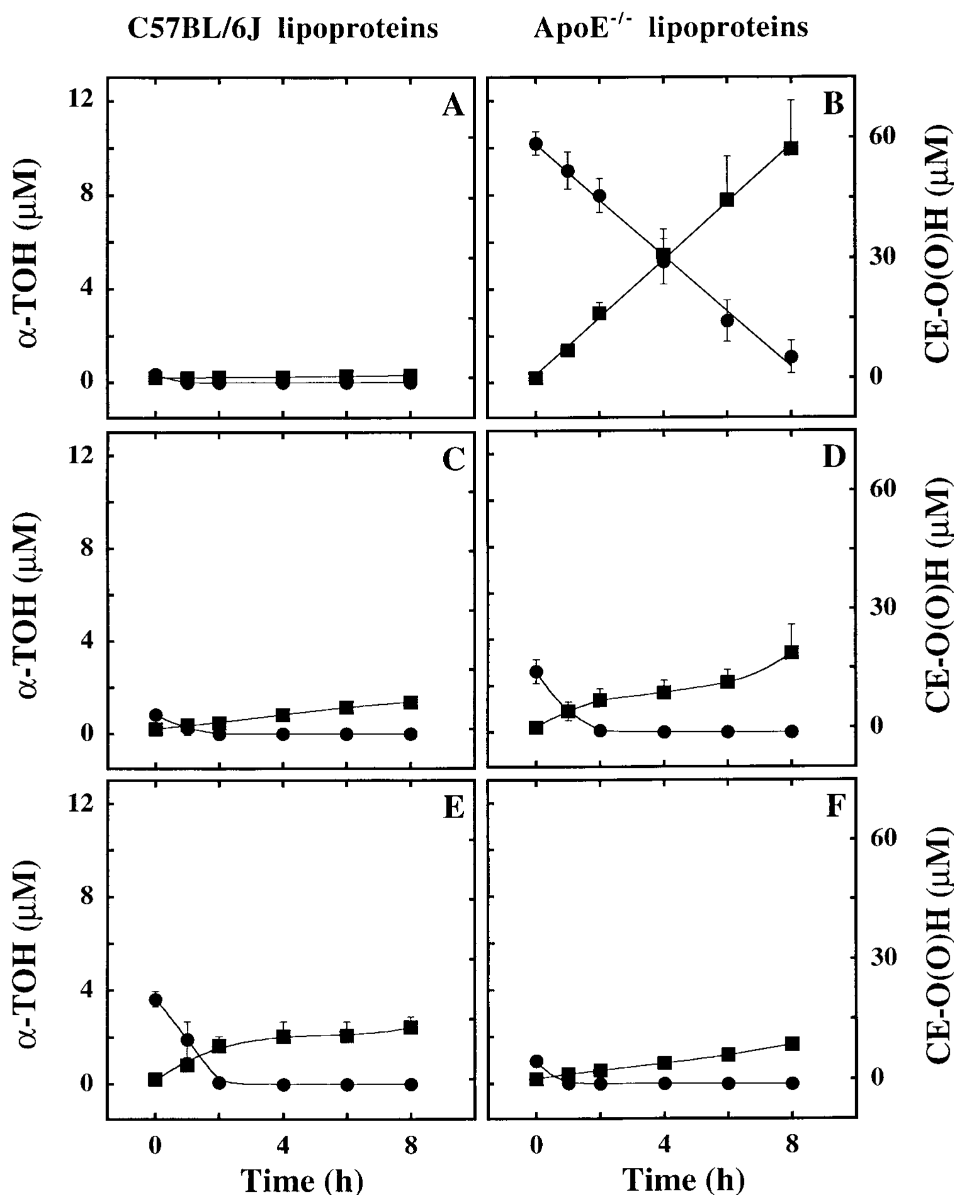

Fig. 7. Lipoproteins isolated from C57BL/6J and apoE$^{-/-}$ mouse plasma exhibit different patterns of oxidizability. M-VLDL (A, B), M-LDL (C, D), and M-HDL (E, F) fractions were isolated from plasma obtained from C57BL/6J (A, C, E) and apoE$^{-/-}$ mice (B, D, F), supplemented with 1 mmol/l AAPH and incubated at 37°C. At the time points indicated, aliquots were withdrawn, extracted, and analyzed for α-TOH (●) and CE-O(O)H (■) as described in Materials and Methods. The concentration of the lipoproteins used (mg total protein per ml) was 0.56 ± 0.06, 0.34 ± 0.06, and 0.85 ± 0.26 for M-VLDL, M-LDL, and M-HDL, respectively, for apoE$^{-/-}$ mouse plasma, and 0.03 ± 0.01, 0.43 ± 0.15, and 1.77 ± 0.74 for M-VLDL, M-LDL, and M-HDL, respectively, for C57BL/6J mouse plasma. These concentrations reflect the situation in the corresponding plasma samples. The data shown are mean values ± SD of three independent experiments carried out in duplicate.

carry out oxidation experiments at a concentration comparable to that used for M-VLDL from apoE$^{-/-}$ mice.

The above results with isolated mouse lipoproteins are compatible with the TMP model developed for human lipoproteins, but appear to contradict the results obtained with apoE$^{-/-}$ mouse plasma oxidation, where $v \leq 1$ was observed irrespective of R_g (see above). To verify an inverse dependence of v on R_i for the isolated M-VLDL derived from apoE$^{-/-}$ plasma, we exposed

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the lipoprotein to either 1 or 0.2 mmol/l AAPH. As shown in **Fig. 8**, and fully consistent with previous results obtained with isolated human LDL and VLDL, similar R_p values were obtained under both oxidizing conditions, although R_i varied greatly. Thus, at constant concentration of M-VLDL, *v* increased from 2.7 to 6.3 upon decrease of the concentration of AAPH from 1 to 0.2 mmol/l. These results demonstrated that the lipids in isolated M-VLDL obtained from apoE$^{-/-}$ mouse plasma oxidized in accordance with TMP when exposed to ROO$^•$.

We therefore examined whether mouse plasma free of low molecular weight compounds (including ascorbate) contains component(s) that could attenuate or prevent TMP. For this, we carried out crossover oxidation experiments in which LPDP prepared from human and apoE$^{-/-}$ mouse plasma was combined with total lipoproteins isolated from either species. Oxidation with 5 mmol/l AAPH of both human and apoE$^{-/-}$ mouse plasma lipoproteins combined with human LPDP (**Figs. 9A**, **B**) gave similar oxidation kinetics with $v \approx 5$. Oxidation of total lipoproteins also gave similar accumulation of CE-O(O)H in the presence of the apoE$^{-/-}$ mouse LPDP, independent of the origin of the total lipoproteins. However, the lipoprotein's α-TOH was depleted more rapidly in the presence of LPDP prepared from apoE$^{-/-}$ mouse plasma, and this was accompanied by accumulation of CE-O(O)H with $v \approx 1$, similar to that seen with the apoE$^{-/-}$ mouse plasma oxidation under two different AAPH concentrations (cf. Fig. 4). These results demonstrated that whereas total lipoproteins from human and apoE$^{-/-}$ mouse plasma oxidized with comparable kinetics, the presence of mouse LPDP decreased *v* when compared with human LPDP.

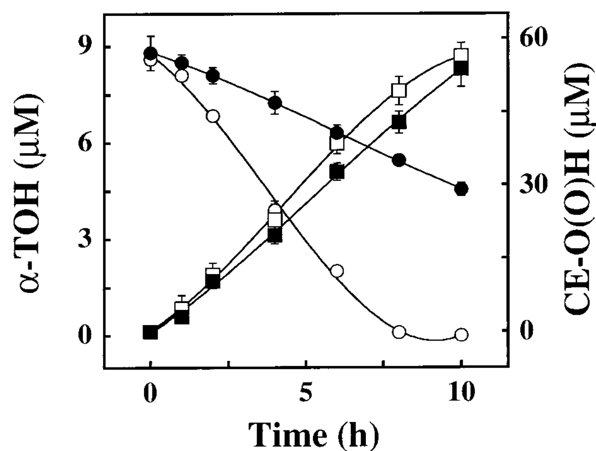

Fig. 8. Radical chain length during the oxidation of isolated apoE$^{-/-}$ mouse M-VLDL increases with decreasing oxidative stress. M-VLDL prepared from the apoE$^{-/-}$ mouse plasma was supplemented with 1 mmol/l (open symbols) or 0.2 mmol/l AAPH (closed symbols) and incubated at 37°C. At the time points indicated, aliquots were withdrawn, extracted, and analyzed for α-TOH (circles) and CE-O(O)H (squares) as described in Materials and Methods. The data shown are mean values ± SD of three separate experiments.

DISCUSSION

In this study we investigated the suitability of apoE$^{-/-}$ mice as an animal model to test TMP by examining whether in vitro lipoprotein and plasma lipid peroxidation in these animals could proceed via TMP. For this, we used ROO$^•$ as the oxidizing agent. We observed that *i*) plasma from apoE$^{-/-}$ mice was readily oxidizable in contrast to that of normal mice; *ii*) M-VLDL of apoE$^{-/-}$ mice was the major carrier of α-TOH and oxidation-susceptible cholesteryl esters, oxidized readily with *v* similar to that found for human LDL, and its relative concentrations of FC, α-TOH, Ch18:2 and Ch20:4 were strikingly similar to those of 'VLDL' isolated from advanced human atherosclerotic plaques; and *iii*) ascorbate-free LPDP of apoE$^{-/-}$ mice reduced the apparent peroxidation chain length, *v* in oxidation experiments using total lipoproteins when compared with human ascorbate-free LPDP.

The results from experiments in which we oxidized plasma prepared from the normolipidemic CBA and C57BL/6J mice revealed a striking resistance to lipid peroxidation when compared to human plasma. Normolipidemic mice are resistant to atherogenesis (47) for a number of reasons. They efficiently metabolize triglyceride-rich chylomicrons, and this includes rapid clearance of β-VLDL via apoE-recognizing receptors, so that the majority of circulating cholesterol is associated with HDL-like lipoproteins. In humans, there is a strong negative correlation between plasma HDL cholesterol and coronary heart disease (48). We have shown previously that human HDL yields substantially lower *v* than isolated LDL or VLDL when exposed to comparable fluxes of peroxyl radicals (46, 49). In addition, on average, only about 1 out of 9 M-HDL particles contains a molecule of α-TOH which makes lipoprotein particles reactive towards aqueous radical oxidants (18). Our present results showing that α-TOH supplementation of normal mouse plasma increases the susceptibility to peroxyl radical-induced lipid peroxidation (after ascorbate consumption) (Fig. 2) is fully consistent with these points and with TMP.

The profile of plasma lipoproteins of the apoE$^{-/-}$ mouse is significantly different from that of normolipidemic mice, with ≈80% of the circulating α-TOH asso-

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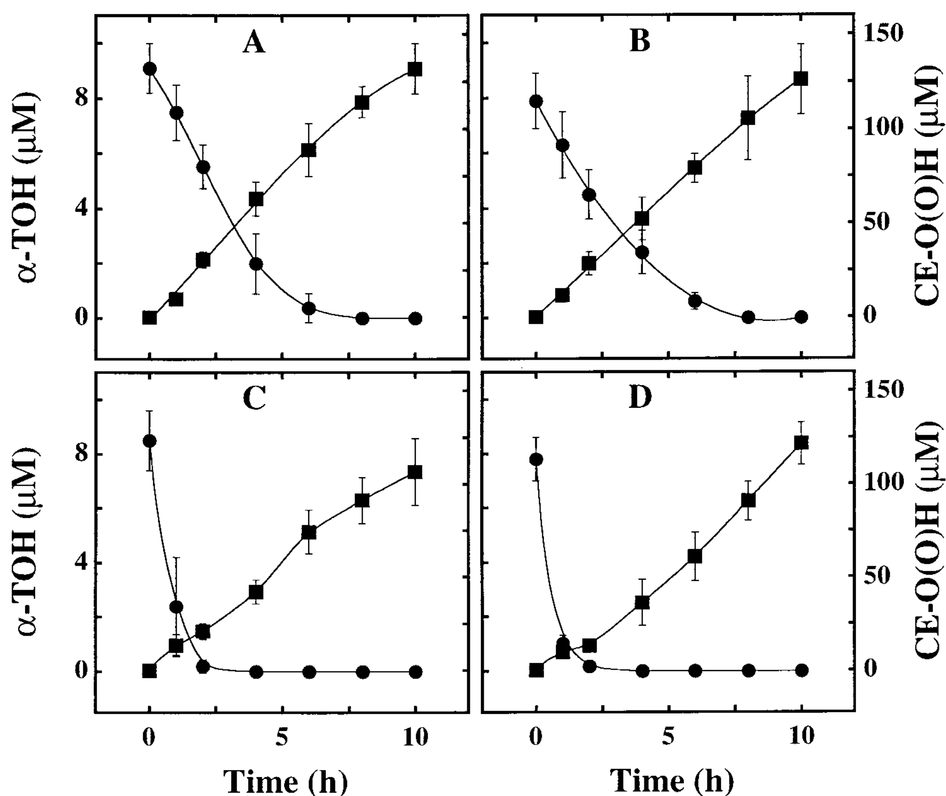

Fig. 9. Mouse lipoprotein-depleted plasma protein fraction retards the formation of CE-O(O)H in human and apo$E^{-/-}$ mouse lipoproteins. Human and apo$E^{-/-}$ mouse LPDP and lipoprotein fractions were prepared as described in Materials and Methods. Human LPDP was combined with either human (A) or mouse lipoproteins (B), and mouse LPDP was combined with either human (C) or mouse lipoproteins (D), as detailed in Materials and Methods. The samples were supplemented with 5 mmol/l AAPH and incubated at 37°C. At the time points indicated, aliquots were taken and analyzed for α-TOH (circles) and CE-O(O)H (squares) as described in Materials and Methods. The data shown are mean values ± SD of three separate experiments.

ciated with the larger lipoproteins (Fig. 5, Table 2), generally considered to be atherogenic. The fact that these more buoyant lipoproteins are the predominant source for oxidizable lipids (Fig. 7) is consistent with the oxidation theory of atherosclerosis. Accordingly, in the atherosclerosis-resistant C57BL/6J mice, almost 80% of the total circulating α-TOH was found in M-HDL, with only 3% associated with M-VLDL. It would be interesting to correlate α-TOH distribution and lipoprotein oxidizability in apo$E^{-/-}$ mice also overexpressing the apolipoprotein A-I gene, as such animals contain lower levels of circulating β-VLDL, higher levels of HDL, and develop atherosclerosis less frequently (50). We would predict plasma from such mice to be intermediate in its susceptibility to lipid oxidation, when compared to that of C57BL/6J and apo$E^{-/-}$ mice (51).

Reminiscent of human plasma, exposure of plasma from the apo$E^{-/-}$ mice to oxidation caused accumulation of substantial amounts of oxidized core lipids in the presence of α-TOH. Also, the co-antioxidant ascorbate was consumed before the onset of detectable lipid peroxidation.

Furthermore, oxidation of M-VLDL isolated from apo$E^{-/-}$ mouse plasma in the presence of α-TOH gave rise to $v > 1$, and v increased with decreasing peroxidation initiation rate, R_i, similar to the situation with human plasma and isolated LDL and VLDL exposed to peroxyl radicals. Together, these results are most readily explained by lipid peroxidation in isolated lipoproteins and plasma of apo$E^{-/-}$ mice proceeding via TMP.

Although plasma from apo$E^{-/-}$ mice is more readily oxidizable than that of normal mice when exposed to ROO$^\bullet$, the v values achieved in the former are lower than those in comparable experiments using human plasma. The reason(s) for this are not known at present. The fact that in apo$E^{-/-}$ mouse plasma exposed to a radical initiator, v appeared to be independent of R_g may indicate the presence of presently unknown com-

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ponent(s) attenuating TMP. Experiments in which we oxidized reconstituted plasma preparations, comprising LPDP from apoE$^{-/-}$ mice, showed initial rapid consumption of α-TOH when compared to human LPDP, independent of the origin of the total lipoproteins. This could be explained if the total peroxyl radical trapping activity of mouse LPDP were lower than that of human LPDP. Importantly, however, such an explanation could not account for the observed independence of v from R_i discussed above. Also, it could not explain the lack of detectable CE-O(O)H in ROO$^•$-induced control mouse plasma (Fig. 1) despite the consumption of α-TOH and hence likely formation of α-TO$^•$. These results could, however, be explained if mouse plasma were to contain a proteinaceous anti-TMP compound. This possibility is intriguing, as to date only non-proteinaceous co-antioxidants are known. Future studies will address this possibility.

In the present study, ROO$^•$ were used throughout as the only (radical) oxidant. As mentioned above, the in vivo oxidant(s) for lipoproteins in the subendothelial space remain unknown, and we do not imply AAPH to be a physiological oxidant. However, the features of AAPH-induced lipoprotein oxidation and its inhibition by antioxidants are, to a greater or lesser extent, also seen with other radical oxidants or conditions that give rise to one electron oxidation reactions. These include l5-lipoxygenases (18, 52), transition metals in the absence and presence of macrophages (53), activated neutrophils (24), the myeloperoxidase–H_2O_2–chloride—tyrosine system (unpublished data), peroxidases (54), and peroxynitrite (55) (S. R. Thomas et al. unpublished results). Therefore, ROO$^•$ derived from AAPH are representative for one-electron oxidants. AAPH and TMP/anti-TMP are, however, not relevant for lipoprotein oxidation induced by nucleophilic oxidants, such as hypochlorite, which oxidizes the lipoprotein independent of α-TOH (56) and which may be important in atherogenesis (6). Also, while lipoprotein (lipid) oxidation has been implicated as a causative factor in atherogenesis, it is clear that other, oxidant-independent, processes also contribute to the disease. For example, apoE$^{-/-}$ mice that are also deficient in macrophage colony-stimulating factor contain higher circulating levels of β-VLDL than apoE$^{-/-}$ mice, yet show dramatically smaller lesion areas than the latter (57).

A striking result observed is the surprising similarity of the lipid profile of the most oxidizable and atherogenic lipoprotein from the apoE$^{-/-}$ mouse plasma (M-VLDL or β-VLDL), and the 'VLDL' fraction isolated from advanced human atherosclerotic plaques. Above all, the relative concentrations of the quantitatively most important oxidation-susceptible cholesteryl esters and α-TOH were very similar. Although the interpretation of this similarity should be treated with caution, this result lends further support to the proposed use of apoE$^{-/-}$ mice as a relevant model for studying the oxidation theory of atherosclerosis. Similarities in plaque development in humans and this mouse model have been suggested (29, 45, 47).

Our results demonstrate that lipids in lipoproteins of apoE$^{-/-}$ mouse plasma can oxidize via TMP, consistent with the oxidation theory of atherogenesis and a link between TMP and lesion development in the apoE$^{-/-}$ mouse. Therefore, we propose apoE$^{-/-}$ mice as an animal model to study the putative role of TMP in the initial stages of in vivo lipoprotein oxidation and atherogenesis, and to use these animals for intervention studies in which anti-TMP agents are tested as potential anti-atherogenic drugs. The observations that *N,N'*-diphenyl 1,4-phenylenediamine is an anti-TMP agent (23) and decreased atherosclerosis in apoE$^{-/-}$ mice (34) are consistent with this proposal.

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