Time-dependent changes to lipids and antioxidants in plasma and aortas of apolipoprotein E knockout mice

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Abstract Oxidation of lipoproteins is thought to be an early event in atherogenesis. To evaluate whether aortic lipoprotein lipid (per)oxidation contributes to atherosclerosis, we investigated the time-dependent changes to lipids and antioxidants in plasma and aortas of apolipoprotein E gene knockout (apoE−/−) mice receiving a high fat diet, and compared these changes with lesion development. Circulating buoyant lipoproteins and associated cholesterol (C), cholesteryl esters (CE), and α-tocopherol (α-TOH) increased within 1 month then remained largely constant up to 6 months. Coenzyme Q (CoQ) remained unchanged for the first 3 months and increased marginally after 6 months. With increasing duration of the diet, plasma lipids showed an increased propensity to undergo peroxyl radical-induced (per)oxidation. Absolute concentrations of aortic C, hydroperoxides and hydroxides of CE (CE-O(O)H) and α-TOH increased gradually while aortic CE increased more markedly with changes to cholesteryl linoleate being most pronounced. Aortic CoQ remained largely unchanged. Overall, the extent of aortic CE (per)oxidation remained low (≤1%) and the ratio of incremental changes of α-TOH to oxidizable lipid remained unchanged. Aortic biochemistry paralleled lesion formation, particularly that in the descending thoracic aorta. Together, our results show that progressing atherosclerosis in apoE−/− mice is associated with increased aortic lipid (per)oxidation as assessed by the concentrations of CE-O(O)H, measured directly by HPLC. This supports the oxidation theory. Measurement of aortic CE-O(O)H may be useful for mechanistic studies studying the relationship between inhibition of in vivo lipid (per)oxidation and atherosclerosis.—Letters, J. M., P. K. Witting, J. K. Christison, A. Weston Eriksson, K. Pettersson, and R. Stocker. **Time-dependent changes to lipids and antioxidants in plasma and aortas of apolipoprotein E knockout mice. *J. Lipid Res.* 1999.** 40: **1104–1112.**

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The oxidation of low density lipoprotein (LDL) in the intima is thought to be an early event in atherogenesis (1). In human atherosclerotic lesions, oxidized proteins (2–7) and lipids (8–10) are present. In the case of lipids, up to 10% of cholesteryl linoleate (C18:2) is oxidized in advanced stages of atherosclerosis (10). LDL-like particles isolated from human lesions and LDL oxidatively modified in vitro have potential pro-atherogenic activities (11). However, at what stage, and to what extent, oxidized lipids accumulate during atherogenesis in the aortas of animal used as models of the disease is unclear.

Lipid peroxidation is thought (1, 12) to contribute to the oxidative modification of LDL's apolipoprotein B-100 through degradation of lipid hydroperoxides (LOOH). LOOH are the primary products formed during the initial stage of in vitro lipoprotein oxidation induced by one-electron oxidants (i.e., radicals) or conditions that give rise to radicals (13). The inhibition of lipid peroxidation by antioxidants is thought to be anti-atherogenic. Therefore, most antioxidants used in animal interventions (14–16), or designed as potential anti-atherogenic agents (17, 18), are radical scavengers that prevent LOOH formation. In contrast to radicals, two-electron oxidants such as hypochlorite preferentially oxidize apolipoprotein B-100 with little direct lipid peroxidation (19, 20). This type of LDL oxidation is not inhibited by typical radical scavengers such as α-tocopherol (α-TOH) (21), although it appears to occur in human atherosclerosis (6, 7, 22).

There is evidence that apoE−/− mice, now commonly used as a model of atherogenesis, represent a useful model for the oxidation theory of atherosclerosis. This is based on the demonstration of oxidation-specific epitopes in lesions and high titres of autoantibodies to malondialdehyde-lysine in serum (23–25). Consistent with this, Hayek et al. (26) and Aviram et al. (27) reported that

Abbreviations: AAPH, 2,2′-azo*bis*(2-amidinopropane) hydrochloride; apoE, apolipoprotein E; apoE−/−, apoE gene knockout; CE, cholesteryl esters; CE-O(O)H, cholesteryl ester hydroxides plus hydroperoxides; C18:2, cholesteryl linoleate; C20:4, cholesteryl arachidonate; C, unesterified cholesterol; HDL, high density lipoproteins; LDL, low density lipoprotein; MSA, mouse serum albumin; DPBS, Dulbecco's phosphate-buffered saline; ROO•, aqueous peroxyl radicals derived from AAPH; TMP, tocopherol-mediated peroxidation; α-TOH, α-tocopherol; VLDL, very low density lipoprotein.

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plasma and lesion lipids in apoE−/− mice are partially oxidized. However, these authors used rather indirect methods to detect oxidized lipids, and the relationship between aortic lipid (per)oxidation and atherosclerosis remains unknown. To facilitate future studies that address a causal relationship between aortic lipid (per)oxidation and atherogenesis, we examined the disease stage-dependent changes in aortic contents of lipids, antioxidants, and oxidized lipids in parallel with a histological assessment of atherosclerosis in apoE−/− mice fed a high fat diet.

MATERIALS AND METHODS

Materials

2,2′-Azo*bis*(2-amidino-propane)-hydrochloride (AAPH) was obtained from Polysciences (Warrington, PA). α-TOH (purity 96%) was obtained as a gift from Henkel Corporation (Sydney, Australia). Cholesteryl linoleate (C18:2) and cholesteryl arachidonate (C20:4), together referred to as cholesteryl esters (CE), unesterified cholesterol (C), ascorbate, formalin, EDTA, and BHT were obtained from Sigma (St. Louis, MO). Coenzyme Q (CoQ) was obtained as a gift from Mitsubishi Chemicals (Japan). 1α, 2α(n)-[^{3}H]-C18:2 ([^{3}H]-C18:2, 48 Ci/mmol) was from DuPont (Boston, MA). [^{3}H]-C18:2 hydroperoxide ([^{3}H]-C18:2-OOH) was prepared by oxidation of [^{3}H]-Ch18:2 (28), and the corresponding [^{3}H]-C18:2 hydroxide ([^{3}H]-C18:2-OH) was obtained by $NaBH_4$ reduction. Authentic C18:2-OOH, used as a standard for CE hydroperoxides and hydroxides (CE-O(O)H), was prepared as described (29) and stored in ethanol at −20°C. Protease inhibitor cocktail tablets were from Boehringer (Mannheim, Germany), and gentamycin was from GIBCO BRL. Chloramphenicol (Sigma) was dissolved in ethanol. Calcium and magnesium chloride-free Dulbecco's phosphate-buffered saline (DPBS, Sigma) was prepared from nanopure water and stored over Chelex-100® (BioRad, Richmond, CA) at 4°C for at least 24 h to remove contaminating transition metals, as verified by the ascorbate autoxidation method (30). The buffer was then filtered and argon-flushed. Organic solvents and all other chemicals used were of the highest quality available.

Animals

Male C57BL/6J mice, homozygous for the disrupted apoE gene (apoE−/−) (23) were purchased from the Jackson Laboratories (Bar Harbor, ME) and then bred at The Heart Research Institute (Sydney, Australia). Mice were housed in groups of 12–14 and fed standard chow (Lab-Feed, Sydney, Australia) until aged 10–11 weeks. Subsequently they were placed for up to 6 months on a diet containing 21.2% fat and 0.15% (w/w) cholesterol (i.e., high fat diet), manufactured by M. J. Hoxey and Associates (Ferndale, Australia) according to specifications of the Harlan Teklad diet TD88137. Animals were fed ad libitum. Periodically, mice were weighed to ensure adequate food intake and to obtain mean body weight. During the course of the study, dermatitis-like skin lesions and murine urological syndrome were noted in some animals as determined by pathology. These conditions appear to be unrelated to atherogenesis. Reducing animal density (from 12–14 to 6–7 animals per 0.93 m^2 cage) notably decreased the progression of, and prevented the new formation of, these pathologies, suggesting behavioral changes due to the consumption of high fat diet. Animals with murine urological syndrome or severe non-xanthomatous skin lesions were excluded, hence the lack of biochemical data for aortas at the 1-month stage. Liver function tests showed no change over the entire course of the study, except a mild increase in circulating AST (data not shown).

Preparation of plasma and lipoproteins

Mice (24–44 g) were anaesthetized by i.p. injection of sodium pentobarbitone (Nembutal®, Boehringer, Ingelheim, Germany) mixed in saline (0.16 mg per 10 g body weight), and the thoracic and abdominal cavities were opened by ventral incision. Blood, taken by cardiac puncture of the left ventricle, was collected into heparinized microvette tubes (Microvette CB 1000S, Sarstedt, Nümbrecht, Germany) and centrifuged immediately (2,500 *g*, 10 min, RT). Aliquots (75 μL) of the resulting plasma from each animal were frozen immediately and stored overnight at −80°C for subsequent lipid and antioxidant analyses as described below for aortic homogenates. Remaining plasma was pooled ($n \geqslant 7$), placed on ice, argon-flushed, and stored (<24 h) at 4°C for oxidation studies, gel filtration, and liver enzyme tests (i.e., ALT, AST, bilirubin, and albumin). Such storage does not alter plasma oxidizability (31, 32).

Removal of aortas for biochemical and histological analyses

After bleeding, mice were gravity-perfused for 5 min with DPBS, containing 5 μm BHT and 2 mm EDTA (buffer A) at near-physiological pressure by inserting a blunt needle into the left ventricle and incising the right atria to allow unrestricted flow. Aortas designated for histology were subsequently perfusion-fixed for 7 min using buffer A containing 4% (v/v) formaldehyde. Aortas used for biochemical analyses were not formalin-fixed, as adventitious oxidation occurs under these conditions as judged by depletion of tissue ascorbate and formation of LOOH, determined by HPLC with post-column chemiluminescence detection (not shown). The hearts, ascending and descending aortas (past the femoral junction) were cleaned under a dissection microscope, excised, and placed immediately in cold buffer A containing one protease inhibitor tablet/150 mL, 0.008% gentamycin, and 0.008% chloramphenicol (for biochemistry) or buffer A containing 4% formaldehyde (for histology). Aortas were carefully cleaned of all extraneous fat under a dissection microscope. Small parts of the intercostal and main arteries were left intact for orientation. For biochemistry, aortas from 7–8 mice were pooled, frozen in buffer A and stored at −80°C. Fixed aortas from each group (⩾5 mice) were transported to ASTRA Hässle (Sweden) for lesion assessment.

Preparation and analysis of aortic homogenates

Pooled aortas were thawed, blotted dry, weighed individually, frozen in liquid N_2, and powderized in a mortar. The fine tissue powder was suspended in 2 mL of cold buffer A, homogenized, extracted, and the organic phase was analyzed for antioxidants, C, CE, and their oxidation products by HPLC (33, 34). Under these conditions, C18:2-OOH and C18:2-OH, the primary oxidized lipids formed in oxidizing lipoproteins of apoE−/− mice (35), eluted with similar retention times. The recovery of CE-O(O)H during work-up of aortic specimens was assessed by addition of [^{3}H]-C18:2-OOH to pooled aortas prior to powderization and subsequent homogenization. Overall recovery of labeled hydroperoxides was 67 ± 2% (mean ± range of two independent studies, each using a pool of 5 whole aortas), with the majority (i.e., 22 ± 2%) of the remaining label eluting with, or close to, authentic [^{3}H]-C18:2-OH (not shown). Thus, we quantified total CE-O(O)H (i.e., C18:2-OOH plus C18:2-OH). To confirm the presence of CE-OOH, HPLC with post-column chemiluminescence detection was used (33). Lipids were quantified by area comparison with standards. Protein was determined using the bicinchoninic acid assay kit (Sigma) with bovine serum albumin as the standard.

Oxidation of plasma

Oxidation of plasma (pooled from 7–14 mice) was performed in duplicate within 24 h of obtaining the samples and using AAPH as the oxidant (34). AAPH yields aqueous peroxyl radicals (ROO•) at a constant rate (36). Antioxidants and lipids were determined as described above for aortic homogenates. For ascorbate, plasma (50 μL) was added to metaphosphoric acid (5% v/v, 50 μL) and frozen on dry ice. Immediately before HPLC analysis, aliquots were thawed and diluted with phosphate buffer (50 μL, 250 mm, pH 7.4), to adjust the pH and ascorbate estimated by HPLC (10).

Size-exclusion chromatography

Samples (300 μL) of argon-flushed, pooled plasma (7–14 mice) were subjected to size-exclusion chromatography, using an FPLC system (Pharmacia) with a Superose-6 (Pharmacia) column (30 × 1.5 cm) eluted with 20 mm sodium phosphate buffer (pH 7.8) at 0.25 mL/min and 4°C. The eluent was monitored at 279 nm and 0.5 mL fractions were collected, pooled (corresponding to the various lipoprotein components), and analyzed for lipids and antioxidants as above. High density lipoprotein (HDL) was collected as the shoulder eluting before mouse serum albumin (MSA).

Preparation of microscopy specimen

For histology, the aortic root and the descending thoracic regions were used. For measurement of atherosclerosis in the aortic root, two cross-sections, 200 and 350 μm distal to where the aortic valve leaflet was judged to first appear, were prepared from dehydrated, paraffin-embedded specimens. Lesion size was determined by planimetry as the intimal area, and the values were averaged for presentation. These areas are directly correlated to the total volume of intima lesion in the aortic root region (A. Weston Eriksson, L. Amrot Fors, K. Pettersson, unpublished results).

For thoracic aortas, a segment (4–5 mm) centered at the third pair of intercostal artery branches in the descending thoracic aortas was dehydrated in ethanol and embedded in paraffin. Ten sections (3 μm thick) were taken 100 μm apart and stained with Weigert's hematoxylin–van Gieson. Intimal thickening was assessed by planimetry using a Leitz DRM microscope with an attached Lucivid device (MicroBrightField, Colchester, VT). This device superimposes a computer-generated display upon the image viewed through a microscope. Planimetry was then performed using Microvid software (MikroMakro AB, Göteborg, Sweden) in a blinded fashion using coded samples. Such an assessment afforded an estimation of the lesion cross-section area by tracing (using a mouse controller) the intimal lesion. Volume estimates were obtained by multiplying cross-section area with 100 μm, and summing the volumes from all 10 sections with intimal lesions. Mean volumes obtained from the same aorta on different occasions varied <5%.

Statistics

The time-dependent changes to biochemical and histological parameters were compared using unpaired Student's *t*-tests with Welch's correction for unequal variances (Prism, Version 2.0, GraphPad Software, San Diego, CA). Statistical difference was accepted at $P < 0.05$.

RESULTS

Histological assessment of atherosclerosis

All mice on the high fat diet gained weight consistently over the 6-month period (not shown). In addition to atherosclerosis (see below), other pathological changes were noted. In particular and at later time points, lesions in carotid, celiac and mesenteric arteries, xanthoma-like lesions in skin, limb edema, and cardiomegaly were observed (not shown). Deposition of lipid-filled cells outside the vasculature is consistent with previous reports on apoE−/− mice (37). Examination of the (transparent) intact aortas showed increasing amounts of white areas of atherosclerotic lesions with increasing duration of receiving the diet. Atherosclerosis in the aortic root (assessed by intimal area) increased almost linearly with time, whereas lesions in the descending aorta (assessed as intimal volume) increased gradually over the first 3 months and then more markedly (**Fig. 1**).

Plasma lipids

Time-dependent changes to plasma lipids and lipid-soluble antioxidants are summarized in **Table 1**. Concentrations of C, C18:2 (the major oxidizable CE), and α-TOH increased significantly with increasing duration of high fat diet, most notably within the first month (Table 1). Despite these marked changes in absolute concentration, C18:2-standardized C and α-TOH did not change over the 6 months (Table 1). The observed ratio of about 1 mol α-TOH per 100 mol C18:2 is typical for plasma lipoproteins, including human LDL (38). Overall, the plasma concentrations of C, C18:2, and α-TOH increased proportionally to each other over the time monitored. In contrast, the absolute concentration of C20:4 did not increase and that of total CoQ (i.e., ubiquinones 9 and 10 plus ubquinols 9 and 10) increased only between 3 and 6 months (Table 1). As a result, α-TOH:CoQ increased during the first 3 months and subsequently decreased again, though it remained higher at 6 months than at Time 0 (Table 1). Small amounts of CE-O(O)H were detected in plasma, and these increased with time, except for a decrease between months 3 and 6 (Table 1). Analyses of extracts from

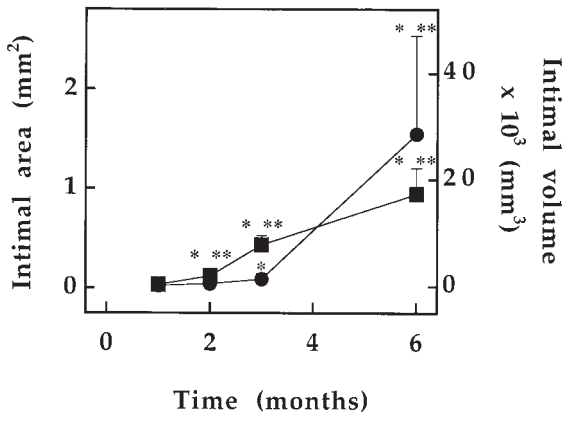

Fig. 1. Histological assessment of the time-dependent changes in lesion size in the aortic root and descending thoracic aortas of apoE−/− mice fed a high fat diet. Intact aortas from individual mice (n = 5 at 1 and 2, n = 12 at 3, and n = 7 at 6 months) were excised, fixed, embedded, and analyzed for lesion size in the aortic root (■) and descending thoracic (●) aortas, as described in Methods. Data shown are mean ± SD. *Denotes a statistical difference from the lesion size assessed after 1 month of diet. **Denotes a statistical difference from preceding time point. Where error bars are not shown, error is smaller than the symbol.

TABLE 1. Time-dependent changes to plasma concentrations of antioxidants and lipids in apoE−/− mice fed a high fat diet

Time	C	C18:2	C20:4	CE[a]	CE-O(O)H	α-TOH	Coenzyme Q[b]
month	*mM*	*mM*	*mM*	*mM*	*μM*	*μM*	*μM*
0	1.8 ± 0.3	1.4 ± 0.3	0.4 ± 0.1	1.8 ± 0.4	0.5 ± 0.1	11.3 ± 2.4	0.23 ± 0.08
1	8.3 ± 1.0[c]	5.0 ± 1.2[c]	0.4 ± 0.2	5.4 ±1.3[c]	0.9 ± 0.4	43.9 ± 10.1[c]	0.16 ± 0.03[c]
2	8.1 ± 0.8[c]	5.4 ± 0.5[c]	0.3 ± 0.1	5.7 ± 0.5[c]	1.1 ± 0.2[c]	45.6 ± 6.4[c]	0.15 ± 0.02[c]
3	9.1 ± 1.4[c]	5.5 ± 0.9[c]	0.5 ± 0.2[d]	5.9 ± 1.1[c]	1.6 ± 0.6[c,d]	51.4 ± 10.7[c]	0.22 ± 0.04[d]
6	8.4 ± 1.3[c]	5.1 ± 1.2[c]	0.3 ± 0.1[d]	5.4 ± 1.3[c]	0.7 ± 0.3[d]	65.1 ± 23.5[c]	0.55 ± 0.11[c,d]

Blood was obtained from individual apoE−/− mice fed a high fat diet for 0, 1, 2, 3, or 6 months and analyzed for the various parameters listed as described in Methods. The data shown represent mean ± SD of 7–15 individual plasma samples taken from different animals.

[a] CE refers to oxidizable lipid (C18:2 + C20:4).

[b] Represents the sum of ubiquinols-9 and -10 plus ubiquinone-9 and -10; no ubiquinol-9 and -10 were detected in plasma.

[c] Denotes value significantly different to corresponding control (0 month) level; $P < 0.05$.

[d] Denotes value significantly different to that of the preceding time point; $P < 0.05$.

3 independent samples of diet showed no detectable CE-O(O)H (data not shown) and negligible CoQ (≤0.025% w/w feed). This indicated that the increase in plasma CE-O(O)H was not simply due to a dietary intake of these oxidized lipids.

Plasma lipoproteins were assessed by gel filtration chromatography before and after the high fat diet (**Fig. 2**). The content of buoyant lipoproteins, previously assigned as very low density lipoprotein (VLDL) (23, 24), increased markedly within the first month and thereafter remained largely unaltered (Fig. 2, inset). Increases in plasma LDL were less pronounced. Changes to HDL were more difficult to assess due to the overlap with the plasma protein peak (referred to as MSA in Fig. 2). This did not affect lipid analyses (see Methods), as plasma proteins are essentially devoid of C, CE, and α-TOH. Overall, the time-dependent changes in VLDL content were similar to those of plasma C, CE, and α-TOH (c.f. Table 1 with Fig. 2). VLDL was the major carrier of plasma lipids and even after 1 month contained 75 ± 7.3% (mean ± SD) of the plasma lipids (**Fig. 3**).

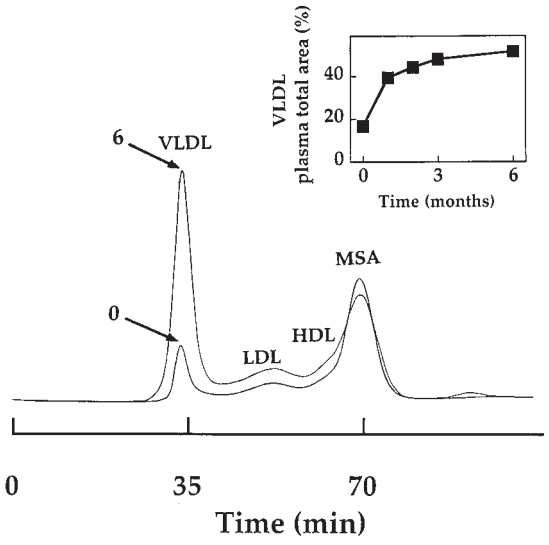

Fig. 2. Time-dependent changes in plasma lipoprotein profile in apoE−/− mice fed a high fat diet. Plasma was collected from individual apoE−/− mice (7–14), pooled and 300 μL was subjected to size exclusion chromatography as described in Methods. Representative chromatograms are shown corresponding to plasma lipid profiles at 0 and 6 months, indicated by '0' and '6', respectively. The inset shows the time-dependent accumulation of plasma VLDL. Data are representative of at least two analyses of independent pooled plasma samples for each time point.

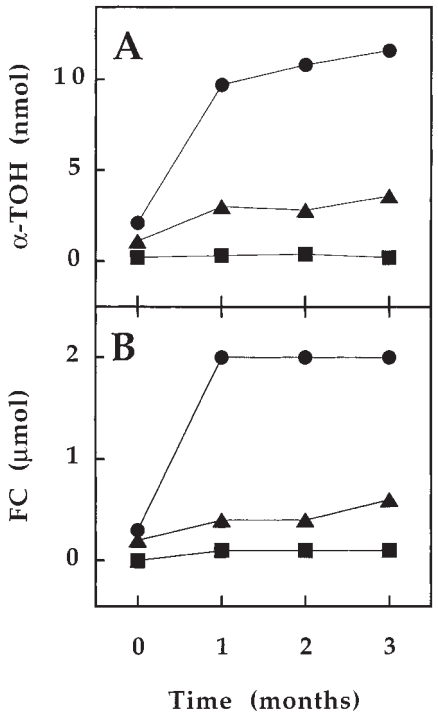

Fig. 3. Time-dependent changes to the content of C and α-TOH in the major lipoprotein fractions prepared from plasma of apoE−/− mice fed a high fat diet. Undiluted, pooled plasma (300 μL) was subjected to size exclusion chromatography. Eluting fractions corresponding to VLDL (●), LDL (▲), and HDL (■) were pooled, extracted, and analyzed for C and α-TOH (panels A and B, respectively) as described in Methods. The data show the total amounts of C and α-TOH present in each class of lipoprotein and are representative of two separate analyses from the same pools of gel-filtered plasma samples. Note the difference in scales in A versus B.

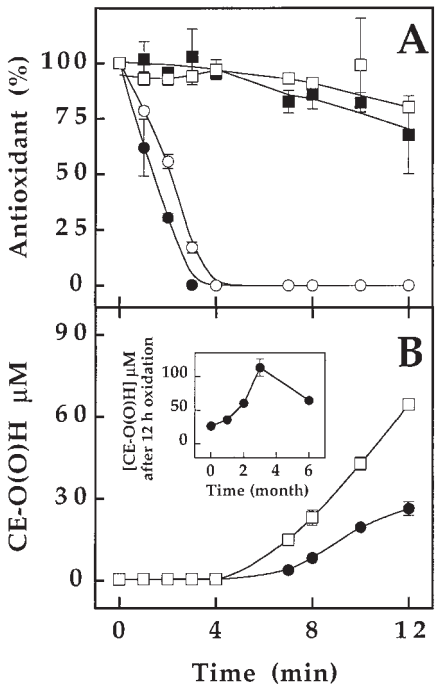

Fig. 4. Time-dependent changes in the ex vivo oxidizability of plasma lipids obtained from apoE−/− mice fed a high fat diet. Pooled plasma obtained from mice fed a high fat diet for 0 (filled symbols) and 6 (open symbols) months was exposed to 5 mm AAPH and incubated under air at 37°C. At the time points indicated, aliquots of the reaction mixture were removed and analyzed for (A) ascorbate (circle) and α-TOH (square) and (B) CE-O(O)H as described in Methods. Inset shows the extent of of CE oxidation after 12 h incubation. Concentration of plasma ascorbate ranged from 49.4 ± 10.2 μm. Initial levels of α-TOH for 0 and 6 months were as in Table 1. Data shown are mean ± range of at least 2 independent plasma oxidation experiments. Where error bars are not shown, error is smaller than the symbol.

Plasma oxidation

Plasma oxidizability was assessed using plasma pooled from groups of mice before (Time 0) and after 1, 2, 3, and 6 months of Western diet by incubating with AAPH. **Figure 4** shows representative data for plasma at 0 and 6 months. CE were protected from peroxidation while ascorbate was present, as shown previously for mouse (35) and human plasma (39). The time of ascorbate depletion in plasma of mice from all time points was similar, i.e., 3.0 ± 0.3 h (mean ± SD), indicating that consumption of the diet did not significantly change plasma ascorbate. After depletion of ascorbate, α-TOH concentration decreased linearly over time (Fig. 4A), and this was associated with an increase in the accumulation of CE-O(O)H (Fig. 4B). The observed formation of CE-O(O)H in the presence of α-TOH is also as shown previously for mouse (35) and human plasma (39) undergoing ROO•-induced oxidation. This can be explained readily on the basis of tocopherol-mediated peroxidation (20). The extent of CE-O(O)H accumulation after 12 h increased during the first 3 months then subsequently decreased (Fig. 4B, inset). The reason for the decrease is not known although it is largely consistent with the time-dependent changes in α-TOH:CoQ (see above). The susceptibility of plasma lipoprotein lipids to peroxidation initiation increases with an increased ratio of α-TOH to CoQ (31). The lipid peroxidation chain length[2] increased consistently from 0.92 to 0.94, 1.03, 3.19, and 3.69 for plasma from the 0, 1, 2, 3, and 6 months time points, respectively. That plasma lipids of apoE−/− mice peroxidize with short chain-length is consistent with previous data (35), although lipids in human plasma peroxidize with comparatively longer chain-lengths (39).

Aortic lipids

To obtain sufficient material 7–8 aortas needed to be pooled, thereby limiting the available data points. Thus, all results were presented as means and statistical analyses were not performed. In general, aortic wet weight increased over the 6 month period (**Table 2**) in parallel with the overall weight of the mice. At Time 0, aortas contained detectable C, C18:2, C20:4, CE-O(O)H, α-TOH, and CoQ, of which C was most abundant. This was particularly apparent when aortic C is expressed per CE or α-TOH and the resulting ratios compared with the corresponding plasma values (compare Time 0 data in Tables 1 and 2). The simplest interpretation of this is that before consumption of the high fat diet, the lipoprotein content in aortas of 12-week-old apoE−/− mice is limited and most of the C is derived from aortic cells. At this stage, α-TOH and CoQ were also present at relatively high concentration, particularly when expressed per C18:2. Thus, we detected 1 mole of α-TOH for every 4 moles of C18:2, and nearly equimolar amounts of vitamin E and CoQ (Table 2). These high ratios of both α-TOH:C18:2 and CoQ:α-TOH contrast with the situation in plasma (see above) and indicate that at this stage most of the aortic vitamin E and CoQ were not located within lipoproteins. Aortas also contained detectable amounts of CE-O(O)H (Table 2), as confirmed by HPLC with post-chemiluminescence detection (not shown).

Upon consumption of the high fat diet, protein-standardized, aortic lipids (Table 2) initially increased slowly when compared with plasma (Table 1). The most dramatic relative increase (⩾80-fold over 6 months) was that of CE (Table 2). This reflected the near absence of CE at Time 0 and is consistent with intimal accumulation of lipoproteins. By comparison, the relative increase in aortic C, α-TOH, and CE-O(O)H was smaller, although C mass increased more than that of CE (Table 2). By contrast, aortic CoQ remained largely unaltered over the 6 months, although there appeared to be an increase between 3 and 6 months (Table 2), similar to the situation in plasma.

The concentrations of protein-standardized aortic CE-O(O)H also increased with time (Table 2). However, when expressed per parent CE, aortic CE-O(O)H decreased from 12.9 (Time 0) to 1.3% (6 months) (**Table 3**). In contrast to plasma, aortic C decreased when expressed

[2] The chain length (v) with which plasma lipids peroxidize is defined here as $v = R_p/R_i$ (where $R_p = d[\text{CE–O(O)H}]/dt$; $R_i = -2d[\alpha\text{-TOH}]/dt$) (43).

TABLE 2. Time-dependent changes to antioxidants and lipids in aortic homogenates prepared from apoE−/− mice fed a high fat diet

Time	Vessel Wet Weight	Protein / Wet Weight	C	C18:2	C20:4	CE[a]	CE-O(O)H	α-TOH	Coenzyme Q[b]
month	*mg*	*mg / mg*		*nmol / mg protein*				*pmol / mg protein*	
0	72	0.02	107.7	1.2	0.7	1.9	24.6	270	244.4
2	100	0.03	84.1	8.4	1.1	9.5	110.8	199	151.9
3	101	0.06	102.4	17.1	3.2	20.3	93.2	400	98.8
6	151	0.03	814.4	95.6	15.8	111.4	191.9	1205	384.2

Entire aortas were removed from apoE−/− mice fed a high fat diet for 0, 2, 3, or 6 months and then analyzed for the parameters listed as described in Methods. The data shown represent mean values of duplicate analyses of a single homogenate (2 mL) obtained from pooled aortas (n = 7–8). All lipid parameters are expressed as nmol per mg protein, except CE-O(O)H, α-TOH, and CoQ (pmol per mg protein).

[a] CE refers to oxidizable lipids (C18:2 + C20:4).

[b] Represents the sum of ubiquinols-9 and -10 plus ubiquinone-9 and -10.

per CE (Table 3). We also expressed the aortic content of α-TOH per CE, because α-TOH primarily protects fatty acyl chains from peroxidation and CE are the predominant source of fatty acids in atherosclerotic lesions (40). CE-standardized α-TOH was lower after than before consumption of the high fat diet, with most of this difference being observed already after 2 months (Table 3). Thereafter the ratio remained unaltered and even after 6 months the molar ratio of α-TOH:CE was 1:80 (Table 3), i.e., higher than that in plasma lipoproteins (see above). The molar ratio of aortic α-TOH to CoQ increased about 3-fold whereas that of CoQ to CE decreased substantially with time (Table 3).

A comparison of the time-dependent changes to the parameters measured is complicated as different sources (e.g., aortic cells versus intimal lipoproteins) contribute differently to individual lipids at different times. Therefore, we also calculated *incremental* changes in aortic lipids to assess the contribution of infiltrated lipoproteins. For this, we expressed the ratio of differences between lipid parameters at different times relative to the Time 0 value. In contrast to their relative expression (Table 3), the ratio of incremental changes in aortic C to C18:2 (i.e., ΔC/ΔC18:2) remained virtually unchanged (**Fig. 5**). This is consistent with the constant C:C18:2 ratio in plasma and indicates that the incremental increase in these lipids indeed reflects the extent of lipoprotein entry into the vessel. Similarly, the ratio of incremental changes in C18:2 to α-TOH (i.e., ΔC18:2/Δα-TOH) remained largely unchanged over the same period (Fig. 5), despite the time-dependent decrease in CE-normalized α-TOH (Table 3). Thus, the content of oxidizable lipid (C18:2) accumulating within the vessel was largely matched by a proportional increase in aortic α-TOH, indicating that there was no deficiency in the content of α-TOH associated with aortic lipoproteins. We also calculated the ratio of incremental changes in C18:2 plus C to (protein-standardized) α-TOH (i.e., [ΔC18:2+ΔC]/Δα-TOH). This ratio increased markedly with time (Fig. 5) consistent with the notion that significant portions of aortic CE become hydrolyzed (40).

TABLE 3. Time-dependent relative changes to aortic lipids and antioxidants in apoE−/− mice fed a high fat diet

Time	$\frac{C}{CE}$	$\frac{CE-O(O)H}{CE}$	$\frac{\alpha\text{-TOH}}{C18:2}$	$\frac{\alpha\text{-TOH}}{CoQ}$	$\frac{CoQ}{CE}$
month					
0	56.7	12.9	0.25	1.2	128.6
2	8.9	11.7	0.02	1.3	16.0
3	5.0	4.6	0.02	4.0	0.5
6	5.6	1.3	0.01	3.1	2.6

Relevant aortic lipid concentrations, expressed per mg protein, were obtained from Table 2. CE refers to oxidizable lipid (C18:2+C20:4).

DISCUSSION

Feeding apoE−/− mice a high fat diet initially caused a rapid then sustained increase to plasma concentrations of buoyant lipoproteins and their associated C, CE, and α-TOH. These marked changes are associated with higher levels of CE-O(O)H and an enhanced propensity of plasma lipids to undergo ex vivo lipid peroxidation. In contrast to the rapid changes observed in plasma, the protein-stan-

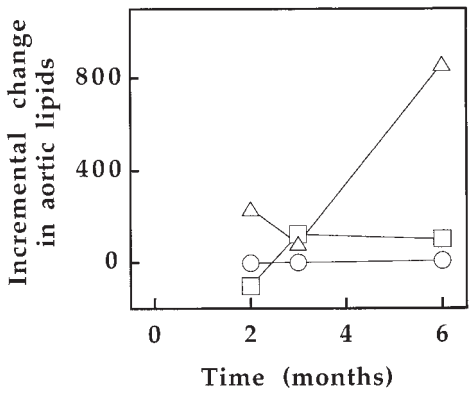

Fig. 5. Time-dependent incremental changes in aortic concentrations of lipids and antioxidants from apoE−/− mice fed a high fat diet for 0–6 months. Aortas from 7–8 mice were obtained, pooled, and analyzed, for the various parameters listed in Table 2, as described in Methods. Incremental changes were calculated as differences between 2, 3, and 6 versus 'Time 0' levels and then expressed as the ratios of C to C18:2 (i.e., ΔC/ΔC18:2, ○), C18:2 to α-TOH (i.e., ΔC18:2/Δα-TOH, □) and [C+C18:2] to α-TOH (i.e., [ΔC+ΔC18:2]/Δα-TOH, △). Ratios were determined from the data shown in Table 2.

dardized content of these lipids increased more gradually in the aortas, more closely reflecting the increase in lesion size in both the aortic root and descending thoracic aortas. The results indicate that these biochemical parameters may be useful, in addition to histology, to assess disease progression in future intervention trials. Also, the persistence of detectable aortic CE-O(O)H may provide a useful tool to directly assess the efficacy with which antioxidants inhibit aortic lipoprotein lipid (per)oxidation and whether this relates to atherosclerosis in this animal model.

A majority of the diet-induced increases in plasma lipids, including α-TOH and CE, were located in VLDL, consistent with previous reports (26, 35, 41, 42). Increased plasma VLDL was associated with an increased propensity of plasma lipids to peroxidize (Fig. 4). Similar to the situation in human plasma (39), lipid peroxidation occurred only after ascorbate consumption, though in the presence of α-TOH. The latter observation is inconsistent with α-TOH acting as a chain-breaking antioxidant, while fully consistent with tocopherol-mediated peroxidation (32–34, 43). The increase in plasma lipid oxidizability may overall be pro-atherogenic (1).

Our results, obtained with a more specific, HPLC-based assay, confirm that plasma of apoE−/− mice contains oxidized lipids (26) and, for the first time, show that these are CE-O(O)H. The concentration of plasma CE-O(O)H increased upon consumption of the high fat diet (Table 1), suggesting that CE-O(O)H could be diet-derived (44). However, the diet used in our study did not contain detectable CE-O(O)H. Furthermore, when administered to rats, dietary lipid hydroperoxides are effectively detoxified in the gut (45). Thus, even if ingested, dietary lipid hydroperoxides may not necessarily give rise to lipid hydroperoxides in blood.

A major aim of this study was to assess whether the onset of atherosclerosis in apoE−/− mice induced by a high fat diet is associated with detectable changes in aortic antioxidants, oxidized and unoxidized lipids. Indeed, the content of aortic CE increased >80-fold over the 6 months monitored (Table 2). This suggests that aortic CE is a suitable and sensitive surrogate for the accumulation of lipoprotein-derived lipids within the vessel wall, particularly as healthy arteries contain little CE (10).

As reported previously (46), lesions were detected earlier in the aortic root than in the descending aorta (Fig. 1). The kinetic of aortic CE accumulation reflected the progression of atherosclerosis, particularly for the lesions in the descending thoracic aorta. This is not surprising given that the aortic root was not included for biochemical analyses. Thus, a limitation for measuring aortic CE as a surrogate sign of atherosclerosis is that the biochemistry and histology measurements were performed in separate aortas. This needs to be considered when comparing data from the two separate procedures. Also, due to the limited amount of material available, it was necessary to pool aortas from several mice. Thus, some 20–25 animals (3 groups of 7–8) alone are required for analyses of the lipid parameters reported here to give three independent values for statistical analyses in future studies.

In contrast to advanced human atherosclerotic lesions (10), the proportion of aortic CE present as CE-O(O)H was substantially smaller in apoE−/− mice after 6 months of high fat diet. This could be because the human specimens studied previously represent advanced lesions, whereas here we used primarily early lesions. This assumes that the content of aortic CE-O(O)H increases with increasing atherosclerosis. Interestingly, however, the ratio of CE-O(O)H to CE *decreased* with increasing duration of diet, indicating that the relative extent of CE oxidation decreased with increasing disease even at the early stages of atherogenesis. This is consistent with a previous report showing that early atherosclerosis is accompanied by a decreased (not increased) accumulation of fatty acid hydroxides in rabbits (47). Thus, we conclude that in apoE−/− mice the extent of oxidation of CE into CE-O(O)H is limited during the early stages of atherosclerosis.

The above conclusion likely extends to the process of aortic lipoprotein lipid (per)oxidation in general. As the content of aortic CE increased with increasing time, a loss of 'substrate' cannot explain the time-dependent decrease in CE-O(O)H:CE. Lipid hydroperoxides such as CE-OOH are labile and subject to conversion to the corresponding hydroxides as well as to the breakdown to secondary lipid peroxidation products such as short chain aldehydes. Importantly, however, our recovery studies showed that most of the CE-OOH remained intact during sample work-up. In addition, we measured both CE-OOH and CE-OH. Therefore, conversion of the hydroperoxide to the more stable hydroxide is not likely to explain why we detected only relatively small amounts of CE-O(O)H. However, at present we cannot exclude the possibility that selective metabolism/removal of CE-O(O)H by enzymes and/or cells in the vessel wall could be involved; e.g., certain cells can remove and detoxify lipoprotein-associated CE-O(O)H (48, 49).

CE-O(O)H can be transient products of in vitro lipoprotein oxidation (50). The observed limited extent of accumulation of CE-O(O)H could therefore be due to their breakdown in aortic tissue. An argument against this is that such breakdown is observed only after a substantial proportion of lipoprotein CE are oxidized (51), which is not the case in the aortas of apoE−/− mice. Also, substantial breakdown of CE-O(O)H occurs only after depletion of α-TOH from lipoproteins (50, 51). Our results show however, that even after 6 months, aortic α-TOH:C18:2 remained higher than that in native (unoxidized) plasma lipoproteins. Perhaps most importantly, the ratio of incremental α-TOH to C18:2 (which most likely reflects the situation of aortic lipoproteins lipids) remained largely unaltered over the duration of the study. Together, the simplest explanation of our data is that in apoE−/− mice, early atherosclerosis is associated with a limited extent of aortic lipoprotein lipid (per)oxidation which itself is not a consequence of α-TOH deficiency. Notwithstanding this, aortic CE-O(O)H can be detected at all stages and increase (in absolute terms) with increasing disease. As CE-O(O)H are possibly formed via tocopherol-mediated peroxidation (35, 43), our present findings further support

the notion (35) that apoE−/− mice may represent a suitable animal model to test a role for tocopherol-mediated peroxidation in atherogenesis, and the prevention of this by co-antioxidants. An initial study in apoE−/−: LDL receptor −/− mice shows that co-antioxidants can indeed inhibit both aortic lipoprotein lipid oxidation and atherosclerosis (34).

Another novel finding of the present study is that aortic CoQ did not increase in parallel with other lipids. Extracellular CoQ, located and transported within lipoproteins, would be expected to enter the vessel wall in the form of lipoproteins. In biological tissues, most of the CoQ is present as ubiquinol (52), a powerful co-antioxidant (31, 53). Present studies are underway to test the potential role of supplemented CoQ on aortic lipoprotein lipid peroxidation and atherosclerosis in apoE−/− mice.

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