Oxidative modification and antioxidant protection of human low density lipoprotein at high and low oxygen partial pressures

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Abstract Oxidative modification of low density lipoprotein (LDL) in the subendothelial space of the arterial wall has been implicated as an initial process in atherosclerosis. In vitro studies of LDL oxidation are usually done at ambient oxygen partial pressure (pO_2; approximately 160 torr, or 21% O_2), which is considerably higher than arterial tissue pO_2 (30–70 torr, and as low as 20 torr, or 2.5% O_2, in atherosclerotic lesions). In addition, β-carotene acts as an efficient free radical scavenger only at low pO_2. Therefore, we investigated the effects of high (20%) and low (2%) pO_2 on the kinetics of LDL oxidation, and the effectiveness of β-carotene compared to other physiological antioxidants in preventing LDL oxidation. At low pO_2, the rate of Cu^{2+}-induced oxidative modification of LDL was lower than at high pO_2. Furthermore, at high pO_2 there was a distinct lag phase preceding the propagation phase of lipid peroxidation in Cu^{2+}-exposed LDL, as measured by cholesteryl ester hydroperoxide formation; in contrast, there appeared to be no distinct lipid peroxidation lag phase in LDL incubated with Cu^{2+} at low pO_2. Elevating α-tocopherol levels in LDL about 5-fold resulted in significant antioxidant protection: the lipid peroxidation lag phase at high pO_2 increased by 45% (from 58 ± 11 to 84 ± 3 min, $P < 0.05$), and the initial rate (0–1 h) of lipid hydroperoxide formation at low pO_2 was reduced by 52% (from 11.6 ± 1.9 to 5.6 ± 1.0 nmol/mg LDL protein/h, $P < 0.01$). In contrast, increasing LDL β-carotene levels about 6-fold did not inhibit LDL oxidation at either pO_2. Most remarkably, low concentrations of ascorbic acid (30 μM) drastically reduced LDL oxidation, regardless of pO_2: the lipid peroxidation lag phase at high pO_2 increased more than 7-fold (from 46 ± 11 min to > 360 min, $P < 0.001$), and at low pO_2 no lipid hydroperoxides could be detected for at least 6 h of incubation. These results show that at low physiological pO_2, Cu^{2+}-induced LDL oxidation occurs at a significantly lower rate than at ambient pO_2. At both high and low pO_2, β-carotene cannot inhibit LDL oxidation, whereas α-tocopherol has a moderate protective effect, and low physiological concentrations of ascorbic acid very strongly suppress LDL oxidation.—**Hatta, A., and B. Frei.** Oxidative modification and antioxidant protection of human low density lipoprotein at high and low oxygen partial pressures. *J. Lipid Res.* 1995. **36:** 2383–2393.

Supplementary key words atherosclerosis • modified lipoproteins • oxygen tension • lipid peroxidation • vitamin C • vitamin E • β-carotene

Oxidatively modified low density lipoprotein (LDL) has a number of biologic properties that are atherogenic. For example, oxidatively modified LDL facilitates foam cell formation from macrophages, is chemotactic for monocytes, T-lymphocytes, and smooth muscle cells, and is cytotoxic (1–3). Based on these biologic properties, LDL oxidation has been implicated as a causal factor in the development of human atherosclerosis (1, 3). As an important corollary of the "oxidative modification hypothesis of atherosclerosis" (1), antioxidants that can prevent LDL oxidation may act as anti-atherogens (2). In the blood stream, LDL oxidation is effectively prevented by numerous water-soluble antioxidants and metal-binding proteins, including ascorbic acid, uric acid, bilirubin, transferrin, and albumin (4, 5). Ascorbic acid appears to be particularly potent in preventing lipid peroxidation in plasma (4). Therefore, it has been postulated that clinically relevant LDL oxidation occurs in the subendothelial space of the arterial wall, where the concentration of some of these antioxidants may be lower than in plasma (1). LDL itself contains a number of lipid-soluble antioxidants, such as tocopherols, carotenoids, and ubiquinols (6, 7). As the lipoprotein content of α-tocopherol and β-carotene is dependent on dietary intake and can be increased by supplementation, much interest in the scientific literature has been directed towards the protection of LDL by these antioxidants.

Numerous studies using in vivo or in vitro supplemen-

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; αt-LDL, α-tocopherol-supplemented LDL; βc-LDL, β-carotene-supplemented LDL; CEOOH, cholesteryl ester hydroperoxides; ct-LDL, control LDL; LDL, low density lipoprotein; pO_2, oxygen partial pressure; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

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tation have found that α-tocopherol, the most abundant antioxidant in LDL, has significant protective effects against LDL oxidation (2, 8–11). In contrast, the effects of β-carotene on LDL oxidation are less clear (2, 10–15). Some epidemiological studies have shown that increased intake of β-carotene or increased adipose tissue β-carotene levels in humans are associated with reduced risk of coronary artery disease (16–18). However, in vivo supplementation of humans with β-carotene does not result in increased resistance of plasma-derived LDL to ex vivo oxidation (10–13), and several in vitro studies also have failed to demonstrate an antioxidant effect of β-carotene against LDL oxidation (2, 12). These experiments with β-carotene-supplemented LDL, with one exception (13), were carried out under air, i.e., at an oxygen partial pressure (PO_2) of about 160 torr (21% O_2). At such high PO_2 levels, β-carotene can be oxidized to β-carotene peroxide and act as a pro-oxidant (19–21). However, the PO_2 in the subendothelial space of the arterial wall, where LDL oxidation likely occurs (1, 3), is considerably lower than in air. Measurements in the arterial wall range from about 30 to 70 torr (4–10% O_2) depending on the location (22); after the onset of atherogenesis, the PO_2 in the subendothelial space may drop even further, corresponding to about 2.5% O_2 (23). At these lower PO_2 levels, LDL oxidation may be slowed considerably, and β-carotene may act as an efficient free radical scavenger.

Therefore, in this study, we investigated LDL oxidation and antioxidant protection at high and low PO_2. The effects of β-carotene under these conditions were studied and compared to the effects of α-tocopherol and ascorbic acid, two other physiological antioxidants. Our data reveal dramatic differences in the kinetics of LDL oxidation and the effects of the various antioxidants tested, depending on the PO_2.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, reduced glutathione, d,l-α-tocopherol, $CuSO_4 \cdot 5H_2O$, isoluminol, and microperoxidase were purchased from Sigma Chemical Co. β-Carotene (all-*trans*) was purchased from Fluka, and Chelex 100 resin from Bio-Rad. Ebselen and 15-hydroperoxy-eicosatetraen-1-oic acid were obtained from Cayman Chemical Co., and Sephadex G-25 was from Pharmacia, Sweden. All other chemicals were of the highest purity commercially available.

LDL isolation

LDL isolation was performed as described by Retsky, Freeman, and Frei (24). Briefly, about 30 ml of blood was collected from a normolipidemic healthy subject using heparinized vacutainer tubes (sodium heparin 286 USP units/15 ml blood). Plasma was obtained by centrifugation and then filtered through a Sephadex G-25 column to remove ascorbic acid and uric acid (24). The plasma density was adjusted to 1.21 g/ml, and a discontinuous gradient was established by layering the plasma under a 0.154 M NaCl solution. The tubes were spun at 80,000 rpm at 7°C for 45 min in an NVT-90 rotor, using a Beckman ultracentrifuge L8-80M (25). LDL was collected from the tubes by sideways puncturing the yellow LDL band. We have shown previously that LDL prepared in this manner contains < 3% albumin contamination (6). Protein concentration in the LDL solutions was measured by the method of Lowry et al. (26), using bovine serum albumin as a standard. Isolated LDL was used immediately for experiments or was stored (not longer than 18 h) under N_2 and at 4°C until used in experiments.

Incubation of LDL at 20% and 2% PO_2

To examine the effects of PO_2 on LDL oxidation, we designed a system to keep the O_2 concentration in the LDL solutions constant, as shown in **Fig. 1.** Chelex-treated phosphate-buffered saline (PBS; 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4) containing 20% or 2% O_2 was prepared by bubbling for 30 min with 20% O_2/80% N_2 or 2% O_2/98% N_2 (Matheson Gas Products, Boston, MA). LDL and Cu^{2+} were then added to these PBS solutions and the PO_2 was maintained constant by performing incubations under a constant gas flow (10–25 ml/min) at 37°C. All gas mixtures used for LDL incubations were first bubbled through distilled water at 37°C to saturate the gas and to avoid concentrating the LDL solutions (Fig. 1). The O_2 concentration in the reaction solutions under the 20% and 2% O_2 gas stream, respectively, were maintained at 7.1 ± 1.4 and 0.4 ± 0.2 ppm (mean ± SD, n = 9) for 24 h, as measured with a dissolved oxygen sensor (DO-6, Presto-Tek, Los Angeles, CA). The expected dissolved O_2 concentration in distilled water at 37°C under air is 6.9 ppm.

Preparation of α-tocopherol- and β-carotene-supplemented LDL

Stock solutions (162.5 μl) of 4.5 mM α-tocopherol in ethanol or 12.8 mM β-carotene in tetrahydrofuran were added to 6.5 ml of plasma and incubated at 37°C under N_2. After incubation for 3 h (α-tocopherol) or 10 min (β-carotene), LDL was isolated as described above. Control LDL was prepared from plasma (6.5 ml) incubated with 162.5 μl of the respective vehicles. LDL isolated from β-carotene- or tetrahydrofuran-treated plasma was incubated for 30 min at 37°C with 0.02 mM ebselen and

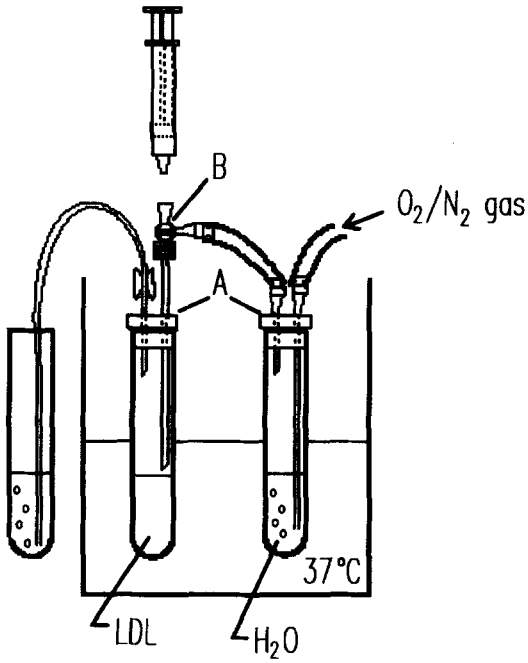


Fig. 1. System for LDL incubation under controlled PO_2 conditions. The O_2 concentration in the LDL incubations was kept constant by blowing 20% O_2/80% N_2 or 2% O_2/98% N_2 gas at a flow rate of 10–25 ml/min on the surface of the LDL solution. The gas was first bubbled through distilled water. The tubes containing the water and the LDL incubations were sealed with a rubber stopper (A) and placed in a thermostatted waterbath (37°C). The needle through which the gas entered the tube containing the LDL solution was connected via a T-valve (B) to a 1-ml syringe. For sample withdrawal, the valve was switched to the position connected to the syringe and the needle was lowered into the LDL solution. Immediately after sample withdrawal, the needle was retracted to the position indicated in the figure and valve B was switched back to the position connected to the gas inlet.

3 mM reduced glutathione to eliminate lipid hydroperoxides formed during the treatment (12). After the incubation, ebselen and glutathione were removed from the LDL solutions by passage through three successive PD-10 columns (Pharmacia) equilibrated and eluted with PBS (12).

LDL oxidation

LDL oxidation was initiated by the addition of 2.5 μM Cu^{2+} to 0.2 mg LDL protein/ml in PBS, followed by incubation at 37°C for up to 24 h. The oxidation of LDL at 20% and 2% PO_2 was carried out in the incubation system described above (Fig. 1). LDL oxidation was characterized by consumption of endogenous α-tocopherol and β-carotene, formation of cholesteryl ester hydroperoxides (CEOOH), and increased anodic electrophoretic mobility of LDL on agarose gels (27).

Some of the LDL preparations used in this study contained residual amounts of KBr. In separate experiments it was determined that the presence of KBr in LDL incubations does not affect the kinetics of Cu^{2+}-induced lipid peroxidation, as assessed by the length of the lag phase and the rate of conjugated diene formation (234 nm absorbance) during the lag and propagation phases (12).

Determination of CEOOH in LDL

To determine the concentration of CEOOH, 250-μl aliquots were withdrawn from the LDL incubations at regular time intervals during the first 6 h of incubation. Aliquots were mixed with 250 μl methanol to precipitate proteins, and neutral lipids were extracted with 2.5 ml hexane that had been washed with water to remove trace amounts of lipid hydroperoxides (28). After shaking vigorously and spinning at 2,000 rpm for 10 min, 2.0 ml of the hexane phase was collected and stored under N_2 gas at -20°C until analysis (within 72 h). Determination of CEOOH was performed by HPLC using an LC-18 column (25 cm × 4.6 mm i.d., Supelco) with post-column chemiluminescence detection (28).

Determination of antioxidants

Aliquots (250 μl) were withdrawn from LDL incubations and the concentrations of antioxidants were determined by HPLC with electrochemical detection (27, 29). The HPLC system was calibrated daily using fresh solutions of d,l-α-tocopherol or β-carotene in ethanol or ascorbic acid in metaphosphoric acid.

Agarose gel electrophoresis of LDL

Gel electrophoresis was performed using the Beckman Paragon electrophoresis system following the manufacturer's instructions. Gels were stained with Sudan Black B.

Data analysis

The length of the lipid peroxidation lag phase in LDL incubated at 20% PO_2 was determined by drawing a tangent to the steepest section of the CEOOH formation curve during the propagation phase and extrapolating to the abscissa; the intersection of the tangent with the abscissa was measured and is presented in the paper as lag phase (min). Lag phases and initial antioxidant levels in control and supplemented LDL preparations were compared using the paired Student's *t*-test or the Mann-Whitney U test where appropriate. Time-dependent changes in antioxidant levels and CEOOH concentrations in LDL were compared using repeated-measures ANOVA. Statistical significance was accepted if the null hypothesis was rejected at the $P = 0.05$ level.

RESULTS

LDL oxidation by Cu^{2+} under ambient, 20%, and 2% PO_2

In order to investigate the effects of PO_2 on the kinetics of LDL oxidation, freshly isolated human LDL (0.2 mg protein/ml, in PBS) was incubated at 37°C with 2.5 μM Cu^{2+} at ambient, 20%, or 2% PO_2. At ambient PO_2 (**Fig. 2A**), α-tocopherol and β-carotene levels in LDL were depleted within 2.5 h of incubation and CEOOH levels increased slowly during this time. After this initial lag phase, the propagation phase of lipid peroxidation ensued, followed by the decomposition of lipid hydroperoxides (Fig. 2A). Incubation of LDL at 20% PO_2 in the apparatus shown in Fig. 1 caused an acceleration of LDL oxidation; the propagation of CEOOH formation occurred earlier than at ambient PO_2 with a lag phase of between 1.0 and 1.5 h (Fig. 2B and **Fig. 3**). Interestingly,

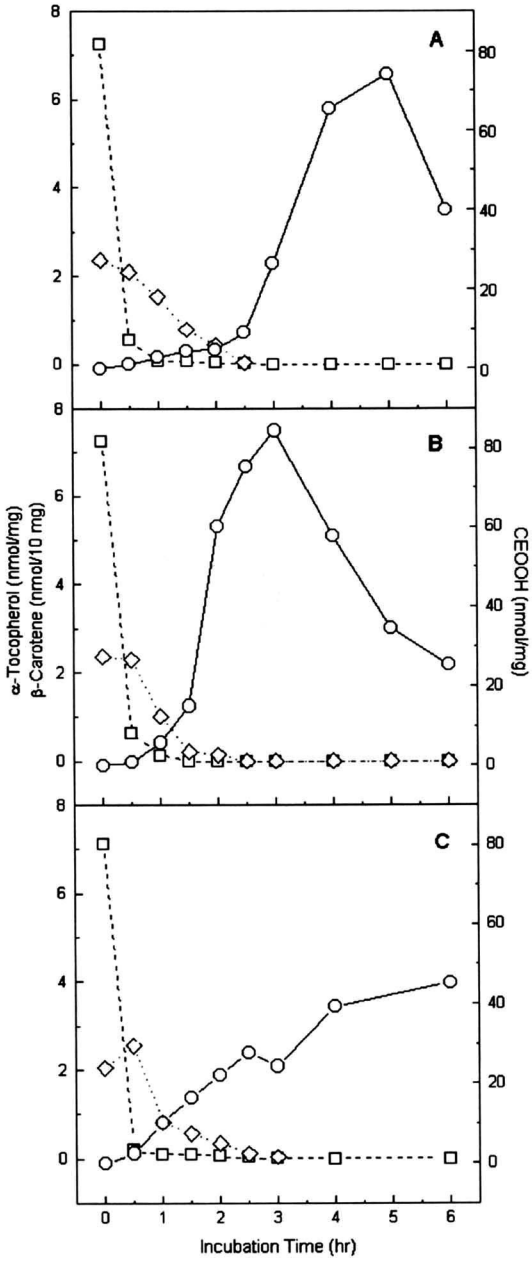


Fig. 2. The effects of PO_2 on Cu^{2+}-induced antioxidant consumption and lipid peroxidation in LDL. LDL (0.2 mg of protein/ml, in PBS) was incubated at 37°C with 2.5 μM Cu^{2+} at ambient (A), 20% (B), or 2% PO_2 (C). At the indicated times, aliquots were withdrawn and the concentrations of α-tocopherol (□), β-carotene (◇), and cholesteryl ester hydroperoxides (CEOOH, ○) were determined. One experiment representative of two is shown.

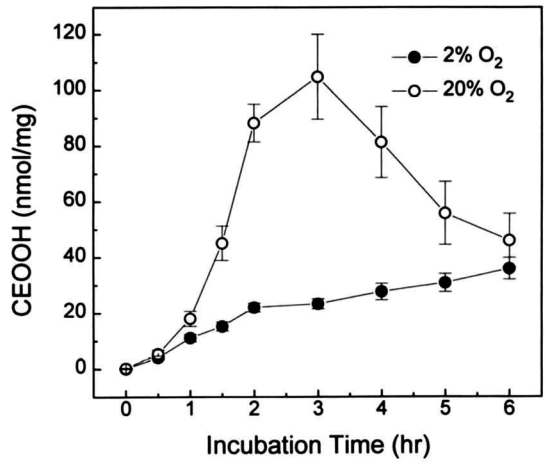


Fig. 3. The effects of PO_2 on Cu^{2+}-induced lipid peroxidation in LDL. LDL (0.2 mg of protein/ml, in PBS) was incubated at 37°C with 2.5 μM Cu^{2+} at 20% (○) or 2% PO_2 (●). At the indicated times, aliquots were withdrawn and the concentrations of cholesteryl ester hydroperoxides (CEOOH) were determined. Data represent the mean ± SEM of ten independent experiments. $P < 0.001$ for the difference between the two time-course curves (0–6 h), using repeated-measures ANOVA.

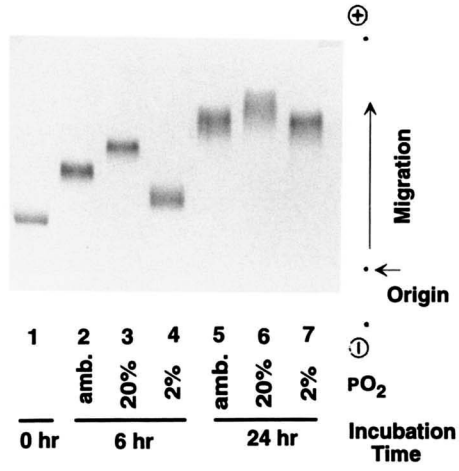


Fig. 4. The effects of PO_2 on electrophoretic mobility of Cu^{2+}-exposed LDL. LDL was incubated as described in the legend of Fig. 2 and aliquots were withdrawn at the indicated times for agarose gel electrophoresis. Lanes from left to right show LDL before incubation (native LDL, lane 1) and LDL incubated at ambient (amb.), 20%, and 2% PO_2, respectively, after 6 h (lanes 2–4) and 24 h incubation (lanes 5–7). One experiment representative of two is shown.

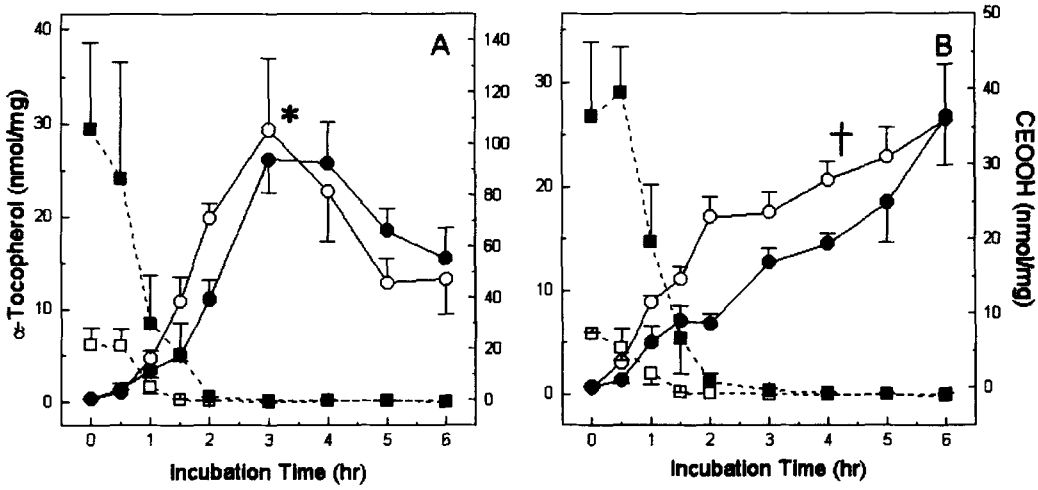


Fig. 5. The effects of α-tocopherol supplementation on Cu^{2+}-induced lipid peroxidation in LDL at high and low pO_2. Control LDL (open symbols) and α-tocopherol-supplemented LDL (closed symbols) (0.2 mg of protein/ml, in PBS) were incubated at 37°C with 2.5 μM Cu^{2+} at 20% (A) or 2% pO_2 (B). At the indicated times, aliquots were withdrawn and the concentrations of α-tocopherol (□, ■) and cholesteryl ester hydroperoxides (CEOOH; ○, ●) were determined. Data represent the mean ± SEM of four independent experiments. *P = 0.056 versus α-tocopherol-supplemented LDL over the first 3 h of incubation (A), and †P < 0.01 versus α-tocopherol-supplemented LDL over 6 h of incubation (B), using repeated-measures ANOVA.

at 2% pO_2 there was no clearly distinguishable lag phase of lipid peroxidation (Figs. 2C and 3). Although the initial rates (0–30 min) of Cu^{2+}-induced CEOOH formation in LDL were similar at 2% and 20% pO_2 (8.1 ± 1.6 and 10.8 ± 2.2 nmol/mg protein/h, respectively; mean ± SD, n = 10), the time course of lipid peroxidation over 6 h of incubation was significantly different (P < 0.001) (Fig. 3). Despite these marked differences in lipid peroxidation, the rates of α-tocopherol and β-carotene consumption in LDL were similar at ambient, 20%, and 2% pO_2 (Fig. 2).

The results on lipid peroxidation were also reflected in the oxidative modification of LDL as assessed by agarose gel electrophoresis. The anodic electrophoretic mobility of LDL incubated for 6 h with Cu^{2+} at 20% pO_2 was greater than that of LDL incubated under air, while the mobility of LDL incubated at 2% pO_2 was only moderately increased (**Fig. 4**). After 24 h of incubation, the electrophoretic mobility of LDL incubated under all three conditions had increased substantially, with LDL incubated at 20% pO_2 being slightly more oxidized than LDL incubated at ambient or 2% pO_2 (Fig. 4).

Effects of α-tocopherol supplementation on LDL oxidation at 20% and 2% pO_2

In order to investigate the effects of α-tocopherol on LDL oxidation at 20% and 2% pO_2, LDL was supplemented in vitro by incubating plasma with an excess of α-tocopherol prior to LDL isolation. This method leads to effective incorporation of α-tocopherol into LDL (30). The changes in α-tocopherol concentration and the effect of α-tocopherol supplementation on CEOOH generation in LDL incubated with Cu^{2+} at 20% pO_2 are shown in **Fig. 5A.** α-Tocopherol-supplemented LDL (αt-LDL) contained about 5 times more α-tocopherol (P < 0.02) than control LDL isolated from vehicle-treated plasma (ct-LDL). This increase in α-tocopherol content was associated with a delay of Cu^{2+}-induced CEOOH generation in LDL. Specifically, the lipid peroxidation lag phase preceding the onset of the propagation phase was significantly increased (P < 0.05) in αt-LDL compared to ct-LDL (**Table 1**), and the difference in the time course of CEOOH formation during the first 3 h of incubation was almost statistically significant (P = 0.056, Fig. 5A).

α-Tocopherol content and CEOOH formation in αt-LDL and ct-LDL incubated with Cu^{2+} at 2% pO_2 are

TABLE 1. Length of the lipid peroxidation lag phase in control and antioxidant-supplemented LDL incubated with Cu^{2+} at 20% pO_2

	Lag Phase (min), mean ± SD		
	Control LDL	Supplemented LDL	P Value
α-Tocopherol (4)	58 ± 11	84 ± 3	0.03
β-Carotene (3)	67 ± 3	60 ± 9	0.29
Ascorbic acid (3)	46 ± 11	> 360	< 0.001

LDL (0.2 mg of protein/ml, in PBS) was incubated at 37°C with 2.5 μM Cu^{2+} at 20% pO_2 and formation of cholesteryl ester hydroperoxides was determined. The data are presented in Figs. 5A, 7A, and 9A, and further experimental details can be found in the legends of these figures. The length of the lipid peroxidation lag phase preceding the onset of the propagation phase was determined as described in Materials and Methods. The numbers in parentheses represent the number of experiments. P values were determined using the paired Student's t-test.

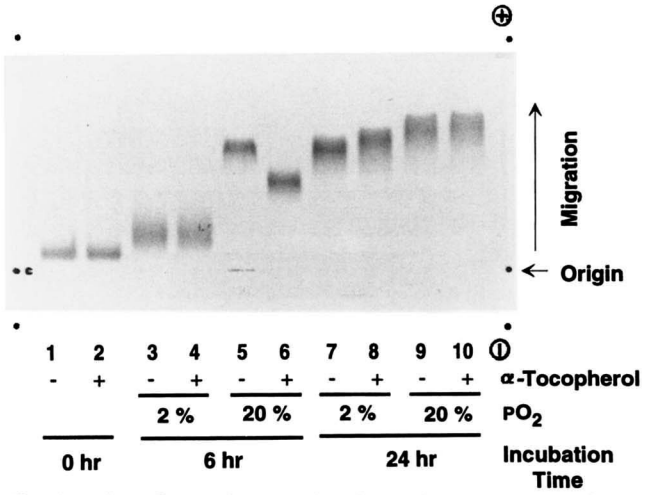


Fig. 6. The effects of α-tocopherol supplementation on electrophoretic mobility of Cu^{2+}-exposed LDL at high and low PO_2. Control LDL and α-tocopherol-supplemented LDL were incubated as described in the legend of Fig. 5 and aliquots were withdrawn at the indicated times for agarose gel electrophoresis. Lanes from left to right show control LDL (-) and α-tocopherol-supplemented LDL (+) before incubation (lanes 1 and 2), after 6 h incubation at 2% (lanes 3 and 4) and 20% PO_2 (lanes 5 and 6), and after 24 h incubation at 2% (lanes 7 and 8) and 20% PO_2 (lanes 9 and 10). One experiment representative of four is shown.

shown in Fig. 5B. As observed above (Figs. 2C and 3), the CEOOH levels in ct-LDL increased steadily over the first 6 h of incubation with apparently no distinct lag phase. α-Tocopherol levels in ct-LDL and αt-LDL were depleted within 1.5 and 2.0 h of incubation, respectively (Fig. 5B), very similar to the rates of α-tocopherol consumption at 20% PO_2 (Fig. 5A). At 2% PO_2, there was significantly less lipid peroxidation ($P < 0.01$) in αt-LDL compared to ct-LDL (Fig. 5B), as also reflected by the initial rates (0–1 h) of CEOOH generation, which were 5.6 ± 1.0 and 11.6 ± 1.9 nmol/mg protein / h (mean ± SD, n = 4) for αt-LDL and ct-LDL, respectively ($P < 0.01$).

Although α-tocopherol supplementation significantly suppressed lipid peroxidation at both PO_2 levels, oxidative modification of LDL as assessed by agarose gel electrophoresis was only moderately affected. The mobility of αt-LDL was lower compared to ct-LDL after 6 h of incubation with Cu^{2+} at 20% PO_2, but no difference in the mobility was observed after 24 h (**Fig. 6**). At 2% PO_2, the mobility of αt-LDL was identical to ct-LDL both after 6 and 24 h of incubation (Fig. 6).

Effects of β-carotene supplementation on LDL oxidation at 20% and 2% PO_2

Plasma LDL was supplemented with β-carotene in vitro as described above for α-tocopherol, leading to an increase in LDL β-carotene content of about 6-fold ($P < 0.02$) compared to ct-LDL. α-Tocopherol levels in β-carotene-supplemented LDL (βc-LDL) and ct-LDL were not significantly different (9.2 ± 2.6 and 8.5 ± 3.0 nmol/mg protein, respectively; mean ± SD, n = 3). βc-LDL and ct-LDL were incubated with Cu^{2+} at 20% or 2% PO_2. Interestingly, although the same ct-LDL and βc-LDL preparations were used in these experiments, the initial β-carotene levels were substantially lower in LDL incubated at 20% compared to 2% PO_2. This may have resulted from a rapid initial loss of β-carotene in the first sample withdrawn from the LDL incubations.

β-Carotene levels in βc-LDL were depleted within 4 h of incubation with Cu^{2+} at 20% PO_2 (**Fig. 7A**), while a small amount of β-carotene remained after 6 h of incubation at 2% PO_2 (Fig. 7B). Lipid peroxidation in LDL

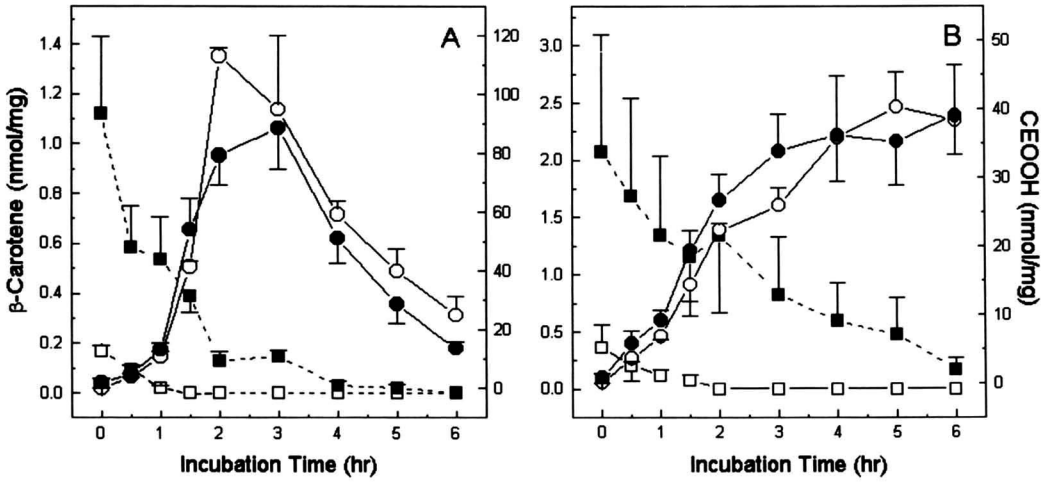
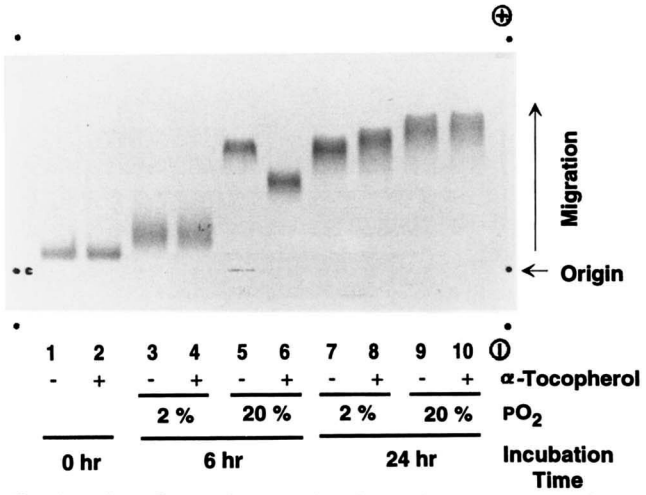


Fig. 7. The effects of β-carotene supplementation on Cu^{2+}-induced lipid peroxidation in LDL at high and low PO_2. Control LDL (open symbols) and β-carotene-supplemented LDL (closed symbols) (0.2 mg of protein/ml, in PBS) were incubated at 37°C with 2.5 μM Cu^{2+} at 20% (A) or 2% PO_2 (B). At the indicated times, aliquots were withdrawn and the concentrations of β-carotene (□, ■) and cholesteryl ester hydroperoxides (CEOOH; ○, ●) were determined. Data represent the mean ± SEM of three independent experiments.



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β-Carotene levels in βc-LDL were depleted within 4 h of incubation with Cu^{2+} at 20% PO_2 (**Fig. 7A**), while a small amount of β-carotene remained after 6 h of incubation at 2% PO_2 (Fig. 7B). Lipid peroxidation in LDL

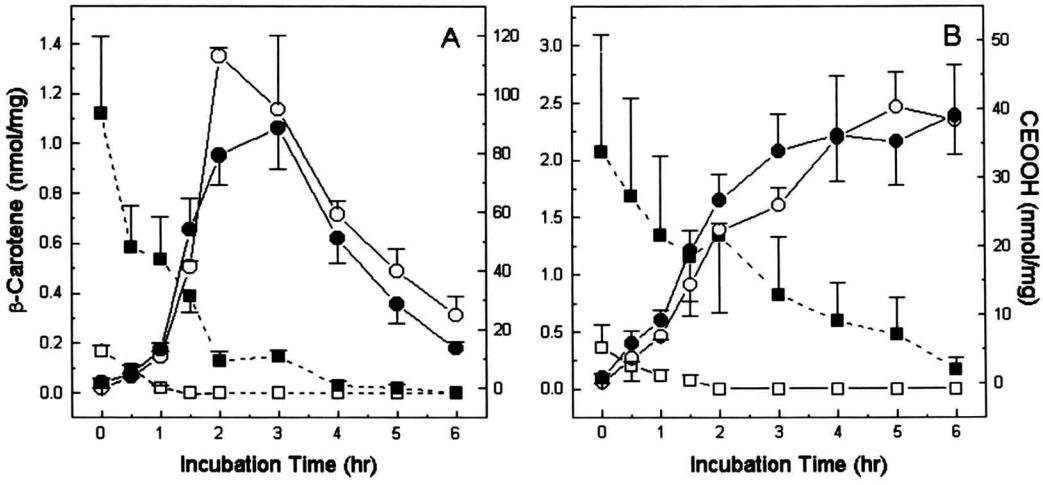

Fig. 7. The effects of β-carotene supplementation on Cu^{2+}-induced lipid peroxidation in LDL at high and low PO_2. Control LDL (open symbols) and β-carotene-supplemented LDL (closed symbols) (0.2 mg of protein/ml, in PBS) were incubated at 37°C with 2.5 μM Cu^{2+} at 20% (A) or 2% PO_2 (B). At the indicated times, aliquots were withdrawn and the concentrations of β-carotene (□, ■) and cholesteryl ester hydroperoxides (CEOOH; ○, ●) were determined. Data represent the mean ± SEM of three independent experiments.

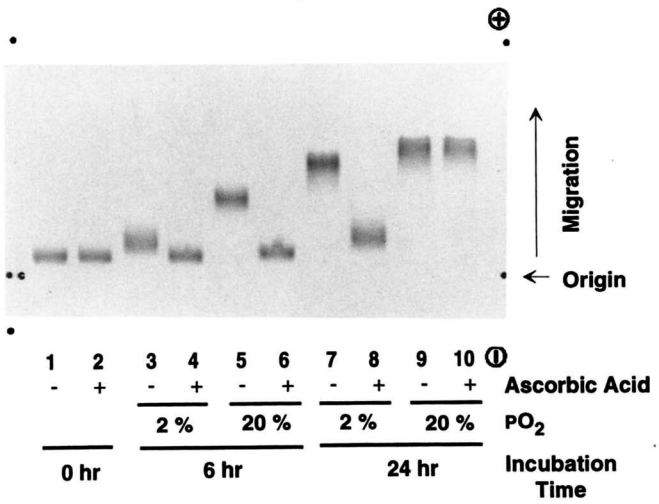

Fig. 10. The effects of ascorbic acid on electrophoretic mobility of Cu^{2+}-exposed LDL at high and low PO_2. LDL was incubated in the absence or presence of ascorbic acid as described in the legend of Fig. 9 and aliquots were withdrawn at the indicated times for agarose gel electrophoresis. Lanes from left to right show LDL incubated in the absence (-) or presence of ascorbic acid (+) before incubation (lanes 1 and 2), after 6 h incubation at 2% (lanes 3 and 4) and 20% PO_2 (lanes 5 and 6), and after 24 h incubation at 2% (lanes 7 and 8) and 20% PO_2 (lanes 9 and 10). One experiment representative of three is shown.

acid was no longer different from ct-LDL, but LDL incubated at 2% PO_2 was still strongly protected by ascorbic acid (Fig. 10).

DISCUSSION

The aims of this study were to investigate oxidative modification of LDL and antioxidant effects of α-tocopherol, β-carotene, and ascorbic acid at an O_2 partial pressure relevant to (patho)physiological conditions in the arterial wall. We first examined the effects of PO_2 on Cu^{2+}-induced oxidation of normal (unsupplemented) LDL. Interestingly, the rate of LDL oxidation was increased at 20% compared to ambient PO_2. This acceleration of LDL oxidation may be related to the different incubation systems used, i.e., LDL incubated under a constant gas flow of 20% O_2/80% N_2 (Fig. 1) versus LDL incubated under air (about 21% O_2, 78% N_2, and 1% argon) in an open system without gas flow. The former compared to the latter conditions may lead to a more enhanced and sustained concentration of dissolved O_2 in the incubations and thus faster LDL oxidation. Oxidative modification of LDL was substantially slower at 2% PO_2 compared to ambient or 20% PO_2. These latter findings are consistent with data reported by Reaven et al. (13) and indicate that the O_2 concentration is a rate-limiting factor for LDL oxidation at low PO_2. This result is not unexpected, as lipid peroxidation is an initial step of LDL oxidation (1, 2, 7) and lipid peroxidation involves the incorporation of O_2 into polyunsaturated fatty acids, i.e., is an O_2-dependent process. In contrast, the rates of α-tocopherol and β-carotene consumption in LDL were independent of PO_2 (see Figs. 2, 5, and 7), indicating that these antioxidants may be consumed by mechanisms other than scavenging of reactive oxygen species and lipid-derived oxygen-centered radicals. For example, at lower oxygen partial pressures, where peroxidation reactions are suppressed (Fig. 3), the LDL-associated antioxidants may primarily react with carbon-centered lipid radicals.

Interestingly, under the incubation conditions used, Cu^{2+}-induced lipid peroxidation in LDL at 2% PO_2 occurred without a clearly distinguishable lag phase. Such a lipid peroxidation lag phase preceding the propagation phase is commonly observed when LDL is oxidized at ambient or 20% PO_2 and has been attributed to the inhibition of lipid peroxidation by LDL-associated antioxidants (7). Although α-tocopherol is by far the most abundant known antioxidant in human LDL, we have shown recently that the tocopherol content of LDL only explains about one third of the length of the lipid peroxidation lag phase in Cu^{2+}-exposed LDL (31). Likewise, Esterbauer et al. (7) have concluded that only about one third of the lag phase in LDL can be attributed to vitamin E, whereas two thirds must be due to other, largely unknown factors. It may be that these unknown factors, in contrast to α-tocopherol, are ineffective at inhibiting LDL lipid peroxidation at 2% PO_2, thus explaining the apparent lack of a lag phase under these conditions. Alternatively, in LDL incubated at 2% PO_2, the rate of lipid peroxidation may not increase after the endogenous antioxidants and other protective factors have been exhausted because the O_2 concentration is rate-limiting (see above). In fact, the initial rates of lipid peroxidation in LDL incubated at 2% and 20% PO_2 were very similar and only later, during the "propagation" phase, was there a greatly increased rate of lipid peroxidation at 20% compared to 2% PO_2 (see Fig. 3). This accelerated formation of lipid hydroperoxides at 20% PO_2 was accompanied by their earlier decomposition and, consequently, a more rapid increase in the electrophoretic mobility of LDL (Fig. 4). The latter is most likely due to apolipoprotein B modification by lipid hydroperoxide breakdown products (1, 2, 7). Our data, therefore, suggest that investigations of LDL oxidation at ambient PO_2 may not reflect the kinetics of LDL oxidation at physiological PO_2 in the subendothelial space of the arterial wall.

We also investigated the protective effects of selected physiological antioxidants against LDL oxidation at high and low PO_2. At 2% PO_2, α-tocopherol supplementation

reduced the initial rate of lipid peroxidation in LDL by more than 50% but the increase in electrophoretic mobility was not suppressed. Similarly, at 20% PO_2, α-tocopherol supplementation led to an increase in the lipid peroxidation lag phase, consistent with published data (7, 8, 10, 30), but electrophoretic mobility was only moderately affected. We did not, however, observe a pro-oxidant effect of α-tocopherol supplementation on Cu^{2+}-induced LDL oxidation under our air incubation conditions. Bowry, Ingold, and Stocker (32), using the same method for lipid hydroperoxide analysis as used in the present study, reported that α-tocopherol supplementation increased, rather than decreased, the initial rate of lipid peroxidation in LDL exposed to aqueous peroxyl radicals generated by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). In support of these data, we recently reported (31) that the tocopherol content of LDL is inversely correlated with the lipid peroxidation lag phase when LDL is exposed to AAPH. In contrast, we found the opposite to be true for Cu^{2+}-induced LDL oxidation, i.e., the higher the lipid-standardized tocopherol content of LDL, the longer the lipid peroxidation lag phase. Taken together, our data indicate that under the specific incubation conditions used by us α-tocopherol exerts a pro-oxidant effect in AAPH-exposed LDL, in contrast to Cu^{2+}-exposed LDL, where α-tocopherol inhibits lipid peroxidation both at high and low PO_2. However, α-tocopherol supplementation appears to offer only moderate or no protection at 20% and 2% PO_2, respectively, against Cu^{2+}-induced increases in electrophoretic mobility of LDL. As increased electrophoretic mobility of LDL correlates well with macrophage degradation and thus increased atherogenicity of LDL (1, 2, 24), these data suggest that α-tocopherol may have little effect on atherogenic modification of LDL in the arterial wall.

Unlike α-tocopherol, β-carotene had no measurable effect on Cu^{2+}-induced lipid peroxidation in LDL at 20% PO_2. These findings are consistent with published data showing that LDL isolated from subjects given β-carotene supplements is not more resistant to Cu^{2+}-induced oxidation than LDL isolated from placebo-treated control subjects (10–13). We have recently confirmed and extended these data by showing that both in vitro and in vivo supplementation with β-carotene does not inhibit LDL lipid peroxidation induced by Cu^{2+} or AAPH (12). However, in almost all the above cited studies, LDL was incubated under ambient PO_2. As β-carotene acts as an efficient antioxidant only at low physiological PO_2 (19, 21), it is conceivable that these studies on LDL oxidation are of limited relevance to physiological conditions and may have overlooked a possible protective effect of β-carotene. Reaven et al. (13) recently addressed this question by incubating LDL from β-carotene-supplemented subjects (containing 19-fold higher levels of β-carotene than LDL isolated from placebo-treated control subjects) at ambient, 4%, and 2% PO_2. No increased resistance to oxidation of β-carotene-enriched LDL compared to control LDL was observed under all incubation conditions (13). Our data using in vitro supplementation with β-carotene (resulting in a 6-fold increased LDL β-carotene content) and incubation at 20% and 2% PO_2 are in agreement with the observations by Reaven and colleagues (13). Therefore, the protective effects of β-carotene against cardiovascular disease (16–18) are most likely not mediated by increased protection of LDL against oxidation in the arterial wall. Alternative mechanisms by which β-carotene may act are reduced capacity of arterial wall cells to modify LDL (13, 33), preservation of endothelial vasodilator function (34), and increased serum high density lipoprotein levels (35).

By far the most dramatic protective effects against Cu^{2+}-induced lipid peroxidation and oxidative modification of LDL both at 20% and 2% PO_2 in the present study were observed in the presence of ascorbic acid. The concentration of ascorbic acid used was only 30 μM, i.e., was at the low end of the physiological concentration range in human plasma (30–150 μM) (5, 29). Using this low concentration of ascorbic acid, the lag phase of lipid peroxidation in Cu^{2+}-exposed LDL at 20% PO_2 was extended more than 7-fold, and the initial rate of lipid peroxidation at 2% PO_2 was reduced by 100%. Interestingly, ascorbic acid was oxidized rapidly and was not present throughout most of the time during which LDL was protected against oxidation. We have shown previously that at ambient PO_2 ascorbic acid oxidation product(s) prevent LDL oxidation, possibly due to selective destruction of metal-binding sites on apolipoprotein B (24). Based on the present findings, these mechanism(s) appear to be relevant at physiological PO_2 as well.

Our data, therefore, suggest that LDL in the subendothelial space of the arterial wall should be strongly protected against metal ion-dependent oxidative modification by ascorbic acid, while α-tocopherol has a moderate effect and β-carotene is ineffectual. Thus, the question arises of whether ascorbic acid is present in the interstitial fluid of the subendothelial space in concentrations sufficient to prevent LDL oxidation. We have recently used a model of human interstitial fluid and shown that in this fluid the ascorbic acid concentration is as high as in plasma (36). It has been shown that in plasma lipid peroxidation is effectively prevented by endogenous ascorbic acid (4, 5, 29). Nevertheless, it may be that ascorbic acid becomes depleted in microenvironments of the arterial wall, where it cannot be replaced readily by passive diffusion from the blood stream. In fact, it is conceivable that the same factors that inhibit

O_2 diffusion into the arterial wall, resulting in low arterial PO_2 levels (22, 23), also limit ascorbic acid diffusion. Therefore, it will be important to investigate whether in vivo ascorbic acid supplementation can inhibit the formation of oxidized LDL in the arterial wall and thus the atherosclerotic process.

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