Resistance of lipoprotein(a) to lipid peroxidation induced by oxygenated free radicals produced by γ radiolysis: a comparison with low-density lipoprotein

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Lipid peroxidation of lipoprotein(a) [Lp(a)] by defined oxygen-centred free radicals ($O_2^{\cdot-}/OH^{\cdot}$, $O_2^{\cdot-}$, $O_2^{\cdot-}/HO_2^{\cdot}$) produced by γ radiolysis was compared with that of paired samples of low-density lipoprotein (LDL). Lp(a) appeared to be more resistant to oxidation than LDL, as indicated by the kinetic study of four markers of lipid peroxidation: decrease in vitamin E, formation of conjugated dienes and aldehydic products, and modification of electrophoretic mobility. In contrast, similar kinetics of lipid peroxidation were obtained for LDL and Lp(a–), which is the lipoparticle issued following the reductive cleavage of apolipoprotein(a) from Lp(a), thus suggesting that the greater resist-

ance of Lp(a) to lipid peroxidation was due to the presence of apolipoprotein(a). Lipid peroxidation of Lp(a) and LDL induced by peroxy radicals, which were produced by an azo compound [2,2'-azobis-(2-amidinopropane) dihydrochloride], confirmed both the resistance of Lp(a) to lipid peroxidation and the propensity of Lp(a–) to exhibit a greater susceptibility to oxidation than intact Lp(a). Our findings also indicated that the high content of apolipoprotein(a) in *N*-acetylneuraminic acid residues was only partly responsible for the resistance of Lp(a) to oxidation.

INTRODUCTION

There is a growing recognition of the importance of lipoprotein(a) [Lp(a)] as an independent risk factor for premature atherosclerotic cardiovascular disease [1,2]. Lp(a) resembles low-density lipoprotein (LDL), the major carrier of plasma cholesterol, and contains 1 mol of apolipoprotein B₁₀₀ (apoB₁₀₀) per particle; it is distinguished from LDL by 1 or 2 mol of apolipoprotein(a) [apo(a)] which is bound to apoB₁₀₀ protein by disulphide linkage [3]. Apo(a) is made up structurally of a genetically determined variable number of repetitive cysteine-rich domains (kringle 4 type 2); its mass was reported to range from 300 to 800 kDa depending on the isoform [4]. Apo(a) is considerably more glycosylated than apoB₁₀₀ (38% versus 5–10%) and contains six-fold more *N*-acetylneuraminic acid [5,6].

The important structural similarities between apo(a) and plasminogen confer a potential thrombogenic action on Lp(a) [7]. On the other hand, the mechanism through which Lp(a) contributes to the acceleration of atherosclerosis remains obscure. *In vitro* experiments showed that Lp(a) may be taken up by human fibroblasts or monocytes via the B/E receptor-mediated pathway, but it is a much poorer ligand than LDL for this receptor, suggesting the occurrence of alternative pathways for removal of these particles from the circulation [8–10]. In particular, recent reports indicated that native Lp(a) and recombinant apo(a) could be bound to macrophages by specific high-affinity receptors [11,12]. In addition, Lp(a) could undergo oxidative modifications that might also account for its atherogenic action, as is well described for LDL [13]. However, whereas a broad knowledge exists of the lipid peroxidation process of LDL, oxidation of Lp(a) is poorly documented and controversial. Lp(a) was found to be less susceptible to copper oxidation *in vitro* than LDL [14]; on the other hand, the susceptibility to

oxidation of Lp(a) was reported to be either decreased [15] or increased [16] in comparison with LDL. Therefore, taking LDL as reference, we aimed to study the lipid peroxidation process induced on Lp(a) by the action of defined oxygen-centred free radicals generated by γ radiolysis of water, which is an appropriate method to produce free radicals selectively and quantitatively. Lipid peroxidation of both Lp(a) and paired samples of LDL by $O_2^{\cdot-}/OH^{\cdot}$, $O_2^{\cdot-}$ or $O_2^{\cdot-}/HO_2^{\cdot}$ free radicals was monitored simultaneously by several parameters. The parameters studied were (i) decrease in endogenous vitamin E concentration, (ii) formation of conjugated dienes, (iii) formation of thiobarbituric acid-reacting substances (TBARS), and (iv) increase in the electrophoretic mobility. We also studied lipid peroxidation of Lp(a) induced by 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), which is an azo compound that generates, at a constant rate, defined peroxy radicals. Resistance of Lp(a) and LDL to AAPH-induced lipid peroxidation was assessed by the determination of the lag phase for the formation of conjugated dienes. Since Lp(a) can be distinguished from LDL by the presence of the apo(a) moiety, we examined the action of both oxygenated free radicals produced by γ radiolysis and peroxy radicals produced by AAPH on Lp(a–) [the lipoparticle obtained by the reductive cleavage of apo(a) from Lp(a)]. We also studied the importance of the high degree of sialylation of apo(a) in the lipid peroxidation process of Lp(a).

EXPERIMENTAL

Isolation of lipoproteins

Blood samples were obtained from five normolipidaemic male donors with high Lp(a) concentrations (0.60–0.80 g/l). Apo(a) phenotyping was assayed according to Gaubatz et al. [17] using

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; apo(a), apolipoprotein(a); apoB₁₀₀, apolipoprotein B₁₀₀; DTT, dithiothreitol; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); Lp(a–), lipoprotein particle obtained upon the reduction and subsequent removal of apo(a) from Lp(a); TBARS, thiobarbituric acid-reacting substances.

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Figure 1 Lipid peroxidation of LDL and Lp(a) by $O_2^{\bullet-}/OH^{\bullet}$ free radicals as a function of the radiation dose

Lipoprotein preparations (0.40 mM phospholipids) were exposed to increasing radiation doses, then taken for assay of vitamin E and lipid peroxidation products as described in the Experimental section: (A) vitamin E, (B) conjugated dienes, (C) TBARS, (D) relative electrophoretic mobility. (■) LDL, (●) Lp(a). Results are means $\pm$ S.D. of three separate preparations.

the Lp(a) phenotype standard from Immuno (Vienna, Austria); phenotypes of the patients were S1, S3, S1/S4, S2/> S4 and S3/> S4 according to the classification of Utermann [4], thus including medium and large apo(a) isoforms. Purification of lipoproteins was performed in the constant presence of EDTA (0.001 M) to prevent spontaneous lipid peroxidation. LDL and Lp(a) were isolated by sequential ultracentrifugation at densities of 1.050 and 1.100 g/ml (180 000 g for 24 h in a Beckman L7

ultracentrifuge); Lp(a) was then separated from LDL by filtration on an agarose gel column using Biogel A-5m (Bio-Rad, Ivry, France) as described previously [18]. Since the protein composition differed between Lp(a) and LDL, lipoprotein concentrations were adjusted to 0.40 mM phospholipid to have identical lipoprotein concentrations. Before irradiation, lipoproteins were dialysed against a 0.01 M sodium phosphate buffer, pH 7.0, for 24 h at 4 °C in the dark to remove EDTA.

Reductive cleavage of lipoproteins

The reductive cleavage was performed as described by Armstrong et al. [18]. Briefly, Lp(a) and LDL (5 mg of total lipoprotein) were incubated for 3 h at 37 °C in 0.02 M Tris/0.15 M NaCl buffer, pH 7.2. One incubation series contained 10 μ M dithiothreitol (DTT), and the second one was incubated without DTT (controls). Lipoprotein preparations were then ultracentrifuged at a density of 1.063 g/ml to remove apo(a), and dialysed as indicated above.

Neuraminidase treatment of lipoproteins

Neuraminidase treatment was performed as described by Sattler et al. [14] with Type X neuraminidase (Sigma). Briefly, Lp(a) and LDL (5 mg total lipoprotein) were incubated for 3 h at 37 °C in 0.1 M Tris buffer, pH 7.2, to give a final volume of 3 ml. One incubation series contained 0.03 unit of neuraminidase/ml the second one was incubated without neuraminidase (controls). Lipoprotein preparations were separated from the enzyme by ultracentrifugation at a density of 1.100 g/ml for Lp(a) or 1.063 g/ml for LDL under the same conditions as for lipoprotein purification, and dialysed as indicated above.

Control of the purity of lipoproteins

The purity of Lp(a) and LDL was checked by agarose gel electrophoresis and by determining the chemical composition; concentrations of the lipid components (total cholesterol, triacylglycerols, phospholipids) were assessed by enzymic methods in a Hitachi 737 analyser (Boehringer Mannheim, Meylan, France). Non-esterified cholesterol was assayed using a kit from Biotrol (Paris, France). Total protein concentration was measured by the Lowry technique using BSA as standard [19].

γ Radiolysis

γ irradiations were performed with a ^{60}Co irradiator (activity: 2.96 TBq); the doses were provided at a rate of 2.7×10^{-2} Gy/s. The irradiations were carried out on 1.2 ml aliquots of lipoprotein preparations. For each experimental set, 1.2 ml samples of non-irradiated lipoprotein preparations [Lp(a) and LDL] were taken as controls. Because of limited Lp(a) accessibility, only a few radiation doses were examined, ranging from 0 to 415 Gy, except for the study of the action of $O_2^{\bullet-}$ radicals, for which the highest radiation dose was 600 Gy. Each experiment was performed in triplicate, i.e. three different lipoprotein preparations issued from any three of our five donors.

Generation of $O_2^{\bullet-}/OH^{\bullet}$ free radicals

Irradiation of the lipoprotein preparations in 0.01 M sodium phosphate buffer at pH 7.0 allowed us to produce simultaneously $O_2^{\bullet-}$ and $OH^{\bullet}$ free radicals with respective yields of 2.8×10^{-7} and 3.4×10^{-7} mol/J (the ratio $[O_2^{\bullet-}]/[OH^{\bullet}]$ is thus close to 1).

Table 1 γ Radiolysis-induced lipid peroxidation of LDL and Lp(a)

Radiation yields for the decrease in vitamin E [G(—Vit.E)] and for the formation of TBARS [G(TBARS)], and values for concentrations of conjugated dienes ([CD]) and TBARS, are given at the highest radiation dose of water γ radiolysis of LDL and Lp(a) preparations.

	Radiation yields ($\times 10^{-7}$ mol/J)		Lipid peroxidation products at the highest radiation dose* (μ M)	
	G(—Vit.E)	G(TBARS)	[CD]	[TBARS]
$O_2^{\cdot-}/OH^{\cdot}$ radicals				
LDL	1.0	1.3	104.4 $\pm$ 21.1	12.9 $\pm$ 1.6
Lp(a)	0.6	—	64.8 $\pm$ 23.0	4.5 $\pm$ 0.9
$O_2^{\cdot-}/HO_2^{\cdot}$ radicals (pH 7.0)				
LDL	0.2	—	< 5	1.1 $\pm$ 0.1
Lp(a)	< 0.1	—	< 5	0.6 $\pm$ 0.1
$O_2^{\cdot-}/HO_2^{\cdot}$ radicals (pH 5.7)				
LDL	1.0	1.4	98.9 $\pm$ 13.1	19.3 $\pm$ 0.6
Lp(a)	0.7	—	64.1 $\pm$ 10.7	13.4 $\pm$ 0.8

* The highest radiation dose was 415 Gy for the study of the action of $O_2^{\cdot-}/OH^{\cdot}$ radicals and of $O_2^{\cdot-}/HO_2^{\cdot}$ radicals at pH 5.7, and 600 Gy for $O_2^{\cdot-}/HO_2^{\cdot}$ radicals at pH 7.0 (see the Experimental section).

Generation of $O_2^{\cdot-}/HO_2^{\cdot}$ free radicals

Prior to irradiation, lipoprotein preparations were dialysed against a 0.01 M sodium phosphate/0.1 M sodium formate buffer; under these conditions, all primary radicals ($OH^{\cdot}$, $H^{\cdot}$, e^-_{aq}) were converted into $O_2^{\cdot-}$ free radicals, with a yield of 6.2×10^{-7} mol/J. The irradiations were performed at pH 7.0, for which the ratio $[O_2^{\cdot-}]/[HO_2^{\cdot}]$ is close to 100, and at pH 5.7, for which the ratio $[O_2^{\cdot-}]/[HO_2^{\cdot}]$ is close to 10.

Markers of lipoprotein oxidation

Vitamin E was assayed by reverse-phase HPLC, with spectrophotometric detection at 292 nm [20]. Conjugated dienes were determined by differential absorbance at 234 nm of the oxidized lipoprotein preparations, taking control preparations as reference, with a 1-mm optical pathway. Conjugated-diene concentrations were calculated using the molar absorption coefficient of $27000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 234 nm [21]. TBARS were measured by a spectrofluorimetric method derived from that of Yagi [22], using the malondialdehyde kit from SOBIODA (Grenoble, France). Relative electrophoretic mobility was determined by agarose electrophoresis of both control and oxidized lipoprotein preparations on a REP analyser (Helena, Saint-Leu, France). Radiation yields for the decrease of vitamin E [G(—vitE)]; initial slope of the curve representing vitamin E concentration as a function of the radiation dose] and for the formation of TBARS [G(TBARS); maximal slope of the curve representing TBARS concentration as a function of the radiation dose] were determined from the estimated curves derived from experimental points, and were expressed in mol of vitamin E lost or TBARS formed per J absorbed respectively. Only G(TBARS) could be determined when the curves for TBARS formation reached a plateau at the highest radiation dose studied.

AAPH-induced lipid peroxidation

EDTA-free lipoprotein preparations ($n = 6$) were diluted in 0.01 M sodium phosphate buffer, pH 7.0, to a final concentration of 0.08 mM phospholipids and oxidation was initiated by addition of aqueous AAPH solution (1 mM final concentration) [23]. Kinetics of oxidation was monitored by the continuous change in 234 nm absorbance at 37 °C for 5 h on a Lambda2 spectrophotometer (Perkin Elmer, Paris, France) equipped with

Perkin-Elmer PECSS software. The absorbance curves of the 1 mM AAPH solution alone and of the lipoprotein solution [LDL or Lp(a)] alone were also determined and subtracted from the lipoprotein oxidation absorbance curves. The lag phase, which indicates the susceptibility of lipoproteins to lipid peroxidation, was defined as the intercept of the tangent of the slope of the absorbance curve in the propagation phase to the baseline, and was expressed in minutes. For comparisons between groups, probability values were calculated by Wilcoxon's sign ranks test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

γ Radiolysis-induced lipid peroxidation of Lp(a) and LDL

Effect of $O_2^{\cdot-}/OH^{\cdot}$ free radicals

γ Irradiation of LDL led to the almost complete consumption of endogenous vitamin E at a radiation dose of 100 Gy; at this dose the vitamin E concentration was only $0.44 \pm 0.08 \mu\text{M}$. The appearance of lipid peroxidation products occurred before a radiation dose of 200 Gy, reaching a plateau as early as the radiation dose of 315 Gy (Figure 1). Table 1 reports the radiation yields for vitamin E decrease and for TBARS formation, and the values for the production of conjugated dienes and TBARS at the highest radiation dose studied (415 Gy). The relative electrophoretic mobility of irradiated LDL increased with respect to the radiation dose, reaching 2.2 ± 0.4 at the highest dose.

The initial content of vitamin E was lower in Lp(a) than in LDL ($3.88 \pm 0.42 \mu\text{M}$ versus $4.97 \pm 0.41 \mu\text{M}$ respectively) and the kinetics of its disappearance were slower; the vitamin E concentration was still $0.77 \pm 0.08 \mu\text{M}$ at a dose of 100 Gy and the initial yield for the vitamin E decrease was about half of that of LDL (Table 1). In the same way, the appearance of lipid peroxidation products was delayed in Lp(a) when compared with LDL, only occurring after a dose of 200 Gy; no plateau was reached at the highest radiation dose (415 Gy) either for conjugated dienes or for TBARS formation. Similarly, we observed a both retarded and smaller modification of relative electrophoretic mobility in irradiated Lp(a) (1.5 ± 0.1 at the highest radiation dose) than in LDL.

The action of $OH^{\cdot}$ radicals, which requires N_2O -saturated lipoprotein preparations, could not be investigated because a

Figure 2 Lipid peroxidation of LDL and Lp(a) by $O_2^{\bullet -}/HO_2^{\bullet}$ free radicals at pH 5.7 as a function of the radiation dose

Lipoprotein preparations (0.40 mM phospholipid) were exposed to increasing radiation doses, then taken for assay of vitamin E and lipid peroxidation products as described in the Experimental section: (A) vitamin E, (B) conjugated dienes, (C) TBARS, (D) relative electrophoretic mobility. (■) LDL, (●) Lp(a). Results are means $\pm$ S.D. of three separate preparations.

clearly visible precipitation of Lp(a) occurred during the N_2O -saturation step.

Effect of $O_2^{\bullet -}/HO_2^{\bullet}$ free radicals

In the presence of sodium formate, γ radiolysis of water led to the formation of $O_2^{\bullet -}$ radicals. The respective proportions of the

Figure 3 Effect of DTT treatment on lipid peroxidation of LDL and Lp(a) by $O_2^{\bullet -}/OH^{\bullet}$ free radicals

DTT-treated (broken lines) and paired control (solid lines) lipoproteins (0.40 mM phospholipids) were exposed to increasing radiation doses, then taken for assay of vitamin E and lipid peroxidation products as described in the Experimental section. (A) Vitamin E, (B) conjugated dienes, (C) TBARS and (D) relative electrophoretic mobility. (■) Control LDL, (□) treated LDL, (●) control Lp(a), (○) treated Lp(a). Results are means $\pm$ S.D. of three separate preparations.

$O_2^{\bullet -}$ radical and its conjugate acid $HO_2^{\bullet}$ vary with pH according to the known equilibrium:

The irradiations were performed at two pH values: pH 7.0, where the $[O_2^{\bullet -}]/[HO_2^{\bullet}]$ ratio is close to 100, at which we could study the action of $O_2^{\bullet -}$ radicals (which are almost the only radical species produced in these conditions); and pH 5.7, where

Table 2 γ Radiolysis-induced lipid peroxidation of DTT-treated LDL and Lp(a)

Radiation yields for the decrease in vitamin E [G(—Vit.E)] and for the formation of TBARS [G(TBARS)], and values for concentrations of conjugated dienes ([CD]) and TBARS are given at the highest radiation dose of water γ radiolysis of DTT-treated LDL and Lp(a) preparations.

	(10 ⁻⁷ × Radiation yields (mol/J))		Lipid peroxidation products at the highest radiation dose* (μ M)	
	G(—Vit.E)	G(TBARS)	[CD]	[TBARS]
$O_2^{\cdot-}/OH^{\cdot}$ radicals				
Control LDL**	1.1	1.4	93.7 ± 11.4	13.8 ± 0.8
Treated LDL	1.2	1.4	96.6 ± 11.1	14.2 ± 0.5
Control Lp(a)**	0.5	—	56.1 ± 10.1	5.1 ± 0.4
Treated Lp(a)	1.0	1.2	87.7 ± 10.5	13.0 ± 0.3
$O_2^{\cdot-}/HO_2^{\cdot}$ radicals (pH 7.0)				
Control LDL**	0.2	—	< 5	1.4 ± 0.2
Treated LDL	0.3	—	< 5	1.5 ± 0.3
Control Lp(a)**	< 0.1	—	< 5	0.7 ± 0.1
Treated Lp(a)	0.2	—	< 5	1.3 ± 0.1
$O_2^{\cdot-}/HO_2^{\cdot}$ radicals (pH 5.7)				
Control LDL**	1.1	1.4	95.6 ± 12.6	18.0 ± 3.3
Treated LDL	1.3	1.1	99.7 ± 13.3	17.8 ± 3.6
Control Lp(a)**	0.7	—	72.2 ± 4.1	10.1 ± 1.2
Treated Lp(a)	1.1	0.6	91.5 ± 8.9	16.2 ± 3.1

* The highest radiation dose was 415 Gy for the study of the action of $O_2^{\cdot-}/OH^{\cdot}$ radicals and of $O_2^{\cdot-}/HO_2^{\cdot}$ radicals at pH 5.7, and 600 Gy for $O_2^{\cdot-}/HO_2^{\cdot}$ radicals at pH 7.0 (see the Experimental section).

** Lipoprotein control preparations were treated in the same conditions as the samples, and thus distinguished from native lipoprotein preparations because of a 3 h incubation period at 37 °C and a supplementary ultracentrifugation step (see the Experimental section).

this ratio is close to 10, at which we could study the action of $HO_2^{\cdot}$ in the presence of $O_2^{\cdot-}$ radicals. The study of the separate action of $HO_2^{\cdot}$ radicals, which requires adjustment of the pH value to 4.0 (the ratio $[O_2^{\cdot-}]/[HO_2^{\cdot}]$ is then close to 0.01), could not be performed because a visible precipitation of Lp(a) occurred at this pH.

Irradiation of LDL at pH 7.0 resulted in a very slow decrease in endogenous vitamin E, as shown by the total disappearance that occurred only at the radiation dose of 450 Gy. TBARS weakly increased after this lag dose and the concentration at the highest radiation dose (600 Gy) was only $1.1 \pm 0.1 \mu$ M (Table 1). At this dose, we did not observe either a formation of conjugated dienes or a noticeable modification of the relative electrophoretic mobility. Lp(a) preparations exhibited still slower kinetics of lipid peroxidation than LDL: vitamin E content at the radiation dose of 450 Gy was still $0.40 \pm 0.11 \mu$ M and initial radiation yield for the decrease of vitamin E was lower (< 0.1 versus 0.2 for Lp(a) versus LDL). The formation of TBARS was also delayed; the value at the highest radiation dose was lower than for LDL (Table 1).

Irradiation of LDL at pH 5.7 led to a complete disappearance of vitamin E at approx. 100 Gy (Figure 2). Formation of conjugated dienes and TBARS occurred at this dose, reaching a plateau at 200 Gy. TBARS production at 415 Gy was as much as $19.3 \pm 1.4 \mu$ M. The relative electrophoretic mobility increased with respect to the radiation dose, being 2.4 ± 0.3 at 415 Gy. On the other hand, Lp(a) preparations exhibited slower kinetics of lipid oxidation than LDL, as indicated by both a smaller decrease of vitamin E (at 100 Gy, vitamin E content of Lp(a) remained at $0.80 \pm 0.39 \mu$ M) and a lower radiation yield for its decrease (0.7 versus 1.0). We also observed both a delayed and lower formation of lipid peroxidation products for Lp(a) than for LDL; the appearance of conjugated dienes and TBARS only occurred at a radiation dose of 200 Gy and no plateau was reached for these products at 415 Gy (Figure 2). Nevertheless, TBARS production

at 415 Gy was important ($13.4 \pm 0.1 \mu$ M) and modification of the relative electrophoretic mobility reached 1.6 ± 0.2 .

γ Radiolysis-induced lipid peroxidation of DTT-treated Lp(a) and LDL

The DTT treatment of lipoproteins required a 3 h incubation step for the lipoprotein preparations with the reductive agent and a supplementary ultracentrifugation step to separate apo(a) from Lp(a—). For all studied free radicals, Lp(a) and LDL control preparations, which were submitted to these steps (except the addition of DTT), exhibited similar kinetics of lipid peroxidation to those of preparations directly submitted to γ irradiation, thus indicating that supplementary steps did not alter consistently the γ radiolysis-induced lipid peroxidation process. In the same way, similar kinetics were obtained for control LDL and DTT-treated LDL, showing that the reductive treatment did not influence the process of LDL lipid peroxidation.

Effect of $O_2^{\cdot-}/OH^{\cdot}$ free radicals

Kinetic parameters for DTT-treated Lp(a) were quite different from those for control Lp(a) and were as follows. The disappearance of vitamin E was early, occurring by the first radiation dose (100 Gy), and the appearance of lipid peroxidation products was effective at a radiation dose of 200 Gy, reaching a plateau between 315 and 415 Gy (Figure 3). Values for conjugated dienes and TBARS at 415 Gy were also higher for Lp(a—) than for control Lp(a) (+56 % and +155 % respectively; Table 2). In the same way, irradiated DTT-treated Lp(a) exhibited a noticeable increase in the electrophoretic mobility, i.e. at a radiation dose of 415 Gy values for relative electrophoretic mobility were 2.0 ± 0.2 and 1.4 ± 0.2 for DTT-treated and non-treated Lp(a).

Figure 4 Effect of DTT treatment on lipid peroxidation of LDL and Lp(a) by $O_2^{\bullet -}/HO_2^{\bullet}$ free radicals at pH 5.7

DTT-treated (broken lines) and paired control (solid lines) lipoproteins (0.40 mM phospholipids) were exposed to increasing radiation doses, then taken for assay of vitamin E and lipid peroxidation products as described in the Experimental section. (A) Vitamin E, (B) conjugated dienes, (C) TBARS, (D) relative electrophoretic mobility. (■) Control LDL, (□) treated LDL, (●) control Lp(a), (○) treated Lp(a). Results are means $\pm$ S.D. of three separate preparations.

Effect of $O_2^{\bullet -}/HO_2^{\bullet}$ free radicals

At pH 7.0, the yield for the vitamin E decrease in DTT-treated Lp(a) was low, although higher than in control Lp(a) (0.2 versus <0.1); TBARS production reached $1.3 \pm 0.1 \mu\text{M}$, thus about twice the value of control preparations (Table 2). We observed neither formation of conjugated dienes nor any noticeable

Figure 5 Effect of neuraminidase treatment on lipid peroxidation of LDL and Lp(a) by $O_2^{\bullet -}/OH^{\bullet}$ free radicals

Neuraminidase-treated (dotted lines) and paired control (solid lines) lipoproteins (0.40 mM phospholipids) were exposed to increasing radiation doses, then taken for assay of vitamin E and lipid peroxidation products as described in the Experimental section. (A) Vitamin E, (B) conjugated dienes, (C) TBARS, (D) relative electrophoretic mobility. (■) Control LDL, (□) treated LDL, (●) control Lp(a), (○) treated Lp(a). Results are means $\pm$ S.D. of three separate preparations.

modification of the relative electrophoretic mobility. At pH 5.7, the disappearance of vitamin E in DTT-treated Lp(a) preparations was early, occurring at a radiation dose of 100 Gy, and the appearance of lipid peroxidation products was effective at this dose, reaching a plateau between 200 and 315 Gy (Figure 4); values for conjugated dienes and TBARS at 415 Gy were higher for DTT-treated Lp(a) (+27% and +60% respectively) than

Table 3 γ Radiolysis-induced lipid peroxidation ($O_2^{\cdot-}/OH^{\cdot}$ radicals) of neuraminidase-treated LDL and Lp(a)

Radiation yields for the decrease in vitamin E [$G(-Vit.E)$] and for the formation of TBARS [$G(TBARS)$], and values for concentrations of conjugated dienes ([CD]) and TBARS, are given at the highest radiation dose (415 Gy) of water γ radiolysis of neuraminidase-treated LDL and Lp(a) preparations.

Neuraminidase treatment	Radiation yields ($\times 10^{-7}$ mol/J)		Lipid peroxidation products at the highest radiation dose (μM)	
	$G(-Vit.E)$	$G(TBARS)$	[CD]	[TBARS]
Control LDL*	1.1	1.5	103.6 ± 17.1	12.7 ± 2.0
Treated LDL	1.0	1.3	111.5 ± 12.2	13.7 ± 1.5
Control Lp(a)*	0.6	—	62.7 ± 13.9	4.8 ± 1.3
Treated Lp(a)	0.8	—	79.3 ± 11.5	6.9 ± 0.7

* Lipoprotein control preparations were treated in the same conditions as the samples, and thus distinguished from native lipoprotein preparations because of a 3 h incubation period at 37 °C and a supplementary ultracentrifugation step (see the Experimental section).

those of control Lp(a) preparations. In the same way, irradiated DTT-treated Lp(a) exhibited an increase in the electrophoretic mobility, i.e. at a radiation dose of 415 Gy values for relative electrophoretic mobility were 1.8 ± 0.2 and 1.4 ± 0.2 for DTT-treated and non-treated Lp(a).

γ Radiolysis-induced lipid peroxidation of neuraminidase-treated Lp(a) and LDL: effect of $O_2^{\cdot-}/OH^{\cdot}$ free radicals

The treatment of LDL by neuraminidase did not lead to consistent changes in the kinetics for either the disappearance of vitamin E or the appearance of lipid peroxidation products (Figure 5). The radiation yields of the disappearance of vitamin E and of the formation of TBARS were quite similar for both treated and control LDL (Table 3). Nevertheless, values for conjugated dienes and TBARS formation at 415 Gy were slightly increased in neuraminidase-treated LDL (+8%; Table 3). Neuraminidase-treated Lp(a) exhibited a greater susceptibility to γ radiolysis-induced oxidation than control Lp(a), as indicated by a faster decrease of vitamin E (Figure 5). Although no plateau was reached at 415 Gy, values for lipid peroxidation products were higher in neuraminidase-treated Lp(a) than those of control Lp(a) (+26% and +44% for conjugated dienes and TBARS respectively). Relative electrophoretic mobility was slightly increased also in neuraminidase-treated Lp(a) [1.7 ± 0.1 versus 1.5 ± 0.1 for control Lp(a)].

AAPH-induced lipid peroxidation of intact and DTT-treated Lp(a) and LDL

Resistance of lipoprotein preparations to AAPH-induced lipid peroxidation was evaluated by the determination of the lag phase for conjugated diene formation. Lp(a) exhibited a significantly longer lag phase than LDL [72.5 ± 9.2 versus 57.7 ± 8.1 min for Lp(a) versus LDL; $P < 0.01$]. DTT treatment of LDL did not modify the lag phase duration: values were 54.2 ± 8.1 and 57.7 ± 8.1 min for DTT-treated LDL and control LDL respectively (not significant). In contrast, values for DTT-treated Lp(a) were significantly decreased in comparison with intact Lp(a) (62.8 ± 6.2 versus 72.5 ± 9.2 min; $P < 0.03$).

DISCUSSION

γ Radiolysis of water is a convenient method with which to study the selective action of oxygen-centred free radicals on lipoproteins [24–26]. The selection of free radical species issued from γ

irradiation of water is obtained by using defined experimental conditions, i.e. N_2O -saturated preparations to select $OH^{\cdot}$ radicals, and 0.1 M sodium formate buffer with defined pH values to select either $O_2^{\cdot-}$ or its protonated form $HO_2^{\cdot}$. Using this method, it was established that oxygen free radicals exhibit different reactivities towards the initiation of the lipid peroxidation process on LDL. In this manner, the oxidizing abilities of oxygen-centred free radicals followed the sequence $HO_2^{\cdot} > OH^{\cdot} > O_2^{\cdot-}$ [24–26]. The results we obtained with LDL preparations confirmed this sequence, even though we studied the actions of $OH^{\cdot}$ and $HO_2^{\cdot}$ radicals only in the presence of superoxide radicals. The precipitation of Lp(a), either during the N_2O -saturation step (required for the selection of $OH^{\cdot}$ radicals) or at a low pH (required for the selection of $HO_2^{\cdot}$ radicals), did not allow us to study the separate action of these two radicals. We hypothesized that both the bubbling of N_2O through Lp(a) preparations (which allowed us to obtain N_2O -saturated lipoprotein solutions) and the low pH resulted in conformational changes of the structure of the Lp(a) lipoparticle which promoted its aggregation and precipitation. The action of $OH^{\cdot}$ and $HO_2^{\cdot}$ radicals on Lp(a) and LDL nevertheless was studied via the results we obtained with $O_2^{\cdot-}/OH^{\cdot}$ or $O_2^{\cdot-}/HO_2^{\cdot}$ free radicals. Given that superoxide radical displays a poor oxidizing ability, the oxidation process we observed with $O_2^{\cdot-}/OH^{\cdot}$ radicals (at a ratio of about 1) and with $O_2^{\cdot-}/HO_2^{\cdot}$ radicals (at a ratio of about 10) can be attributable, for the most part, to the action of $OH^{\cdot}$ and of $HO_2^{\cdot}$ respectively. Besides, the kinetics of LDL lipid peroxidation that we observed with these more complex systems were similar to those in other reports that discussed the separate actions of these radicals [24–26].

Our findings showed that Lp(a) was more resistant to lipid peroxidation than LDL, as indicated by both a slower disappearance of endogenous vitamin E and a slower formation of lipid peroxidation products (conjugated dienes and TBARS). Oxidizing abilities of the three radicals studied followed the same sequence for Lp(a) and for LDL, i.e. $HO_2^{\cdot} > OH^{\cdot} > O_2^{\cdot-}$. In all these experiments, Lp(a) was found to be more resistant to γ radiolysis-induced lipid peroxidation than paired LDL. The oxidation experiments we performed with AAPH also showed a greater resistance of Lp(a) to oxidation than LDL. Thus, using four different radical species (superoxide, hydroxyl, hydroperoxyl and peroxy radicals) we found Lp(a) to be more resistant to lipid peroxidation than LDL. Our results are in agreement with those of Sattler et al. who reported a longer lag phase for conjugated diene formation during copper-ion-induced lipid peroxidation for Lp(a) than for LDL [14].

On account of the resistance of Lp(a) to lipid peroxidation, the lower initial content of vitamin E in Lp(a) than in LDL may appear surprising. This result was also observed by others [14,16]. It is worth noting that vitamin E, though quantitatively the most important lipophilic antioxidant in both LDL and Lp(a) [27,28], was shown to be only partially responsible for the resistance of the lipoproteins to lipid peroxidation. Indeed, numerous studies reported a lack of correlation between the initial endogenous vitamin E content and the lag phase for conjugated diene formation during copper-induced oxidation of LDL preparations [13,29,30]. It was suggested that other antioxidant molecules present in the lipoproteins may play an important role in the susceptibility of lipoproteins to lipid peroxidation [31,32].

Lp(a-), the lipoprotein particle obtained upon the reductive cleavage of Lp(a), exhibited similar susceptibility to that of LDL to both γ radiolysis-induced and AAPH-induced lipid peroxidation, thus suggesting that the greater resistance of Lp(a) to *in vitro* lipid peroxidation was due to the presence of the apo(a) on the lipoparticle. The removal of apo(a) from Lp(a) also led to similar values for the formation of lipid peroxidation products in Lp(a-) and in LDL, which is consistent with the observation that Lp(a-) had both similar structure and chemical composition to LDL [3,14]. As a consequence of the protective effect of apo(a) towards lipid peroxidation, it is conceivable that the size of apo(a) may exert an effect on the extent of the resistance of Lp(a) to oxidation: a small isoform of apo(a) may be less protective than a large isoform towards free radical attack of the lipid moiety of Lp(a). In our study, Lp(a) preparations were obtained from patients with different apo(a) phenotypes, including medium and large isoforms. With a low and similar dispersion for Lp(a) and LDL, all experiments pointed both to a greater resistance of Lp(a) than LDL to lipid peroxidation, and to the propensity of Lp(a-) to be more susceptible to oxidation than Lp(a), thus suggesting that the size of apo(a) is not of prime importance in the protection of the lipid core of Lp(a) against lipid peroxidation. On the other hand, we showed that neuraminidase treatment of Lp(a) did not modify consistently the kinetics of lipid peroxidation induced by free radicals generated by γ radiolysis, indicating that the high degree of sialylation of apo(a) is only partly responsible for the resistance of Lp(a) to oxidation. This result, however, disagrees with the report of Sattler et al., who suggested that the removal of just the neuraminic acid residues of Lp(a) was sufficient to reduce its resistance to oxidation to that of LDL [14].

The greater resistance of Lp(a) to lipid peroxidation compared with LDL could appear inconsistent with its proatherogenic properties. Nevertheless, the oxidative modification of Lp(a) seems to play a role in the recognition of Lp(a) by cellular types involved in the development of atherogenesis. *In vitro* modification of Lp(a) by malondialdehyde or by copper ion oxidation was found to abolish its recognition by the LDL receptor, but to induce its uptake by the scavenger receptor of human monocytes/macrophages [9,16]. On the other hand, current evidence indicates that pathophysiologically important oxidation processes only occur after retention of the lipoproteins within the arterial intima [33]. Lp(a) was demonstrated to bind to arterial proteoglycans and glycosaminoglycans with greater affinity and capacity than does LDL [34]. Its retention in intima, as complexes with the extracellular matrix components [which leads to the formation of Lp(a) aggregates], would increase the probability of

it undergoing oxidative modifications; in spite of a greater resistance to lipid peroxidation, immobilized Lp(a) will finally become oxidized and thus participate in the development of atherosclerotic lesions.

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