Major differences in oxysterol formation in human low density lipoproteins (LDLs) oxidized by $^{\bullet}OH/O_2^{\bullet-}$ free radicals or by copper

Stéphanie Zarev[a,b], Patrice Thérond[b], Dominique Bonnefont-Rousselot[a,*], Jean-Louis Beaudeux[a], Monique Gardès-Albert[c], Alain Legrand[a,b]

[a] *Laboratoire de Biochimie Métabolique et Clinique, Faculté de Pharmacie, 4, avenue de l'Observatoire, 75270 Paris Cedex 06, France*
[b] *Laboratoire de Biochimie, Hôpital de Bicêtre, 78 rue du Général Leclerc, 94275 Le Kremlin Bicêtre Cedex, France*
[c] *Laboratoire de Chimie-Physique, URA 400 CNRS, 45, rue des Saints-Pères, 75270 Paris Cedex 06, France*

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Abstract The aim of our study was to determine the oxysterol formation in low density lipoproteins (LDLs) oxidized by defined oxygen free radicals ($^{\bullet}OH/O_2^{\bullet-}$). This was compared to the oxysterol produced upon the classical copper oxidation procedure. The results showed a markedly lower formation of oxysterols induced by $^{\bullet}OH/O_2^{\bullet-}$ free radicals than by copper and thus suggested a poor ability of these radicals to initiate cholesterol oxidation in LDLs. Moreover, the molecular species of cholesteryl ester hydroperoxides produced by LDL copper oxidation seemed more labile than those formed upon $^{\bullet}OH/O_2^{\bullet-}$-induced oxidation, probably due to their degradation by reaction with copper ions.

Key words: Copper; Free radical; Hydroperoxide; Lipoprotein; Low density lipoprotein; Oxidation; Oxysterol

1. Introduction

Oxidative modifications of human low density lipoproteins (LDLs) are believed to play a key role in the initiation and the development of atherosclerotic plaques [1,2]. Such oxidative modifications are often reproduced in vitro with a copper oxidation [3,4]. Nevertheless, the copper-mediated oxidation mechanism that could explain oxidative attack of LDL is not well defined, and the involvement of copper in the very first steps of LDL oxidation is still a subject of discussion [5,6]. In vivo, several enzymatic or non-enzymatic mechanisms have also been proposed for the initiation of LDL peroxidation. Among them, oxygen free radicals produced by macrophages and vascular cells (especially $^{\bullet}OH$ and $O_2^{\bullet-}$ free radicals) have been reported to play a role in the initiation of the oxidative process [7]. LDLs oxidized by copper or by defined oxygen free radicals could exhibit different biological properties [8], due to quantitative and/or qualitative discrepancies in the lipid peroxidation products generated in each case. Therefore, in this study, two methods have been compared with regard to their ability to oxidize LDLs, namely copper oxidation and water γ-radiolysis. This latter method allows a selective production of oxygen free radicals, especially $^{\bullet}OH$ and $O_2^{\bullet-}$, with well-known radiolytic yields (number of free radicals produced per unit of energy absorbed, that is per Joule). γ-Radiolysis has been previously used to initiate the peroxidation of human LDLs [9,10] and high density lipoproteins [11] and allowed us to propose schemes for the kinetics of the degradation of these lipoproteins under free radical attack.

Among the biological properties of oxidized LDLs, their cytotoxicity to different cell types (especially endothelial and smooth muscle cells) has been widely described [12,13]. Several studies showed a good correlation between the cytotoxicity of oxidized LDLs and their extent of lipid peroxidation, as measured by the concentration of thiobarbituric acid-reactive substances (TBARS) in LDLs [7]. This cytotoxicity could be mimicked by a lipid extract of the oxidized LDLs [7,12], thus suggesting that some products of lipid peroxidation may be the cytotoxic intermediates. Among these products, oxysterols have been suggested to be highly involved in the cytotoxicity of oxidized LDLs [14–19].

The aim of our study was thus to determine the oxysterol formation in LDLs oxidized by $^{\bullet}OH$ and $O_2^{\bullet-}$ free radicals and to compare this formation with that obtained by a copper oxidation, when a similar level of lipid peroxidation has been reached. This level was assessed by assaying the concentration of conjugated dienes and TBARS formed in LDLs upon oxidation. Three oxysterols were quantified, namely 7-ketocholesterol (7-KC), 7β-hydroxycholesterol (7-OH) and 5,6β-epoxycholesterol (5,6β-epoxy), by gas chromatography, both in the esterified and in the unesterified cholesterol fractions. The results obtained were interpreted as a function of the consumption of the cholesteryl esters and of the formation of the cholesteryl ester hydroperoxides from linoleic and arachidonic acids, assayed by high performance liquid chromatography (HPLC). To our knowledge, this was the first time that oxysterol and cholesteryl ester hydroperoxide formation was measured in LDLs oxidized by well-defined oxygen free radicals ($^{\bullet}OH/O_2^{\bullet-}$) and compared with that of copper-oxidized LDLs.

2. Materials and methods

2.1. Isolation of LDLs

LDLs ($1.019 < d < 1.063$) were isolated by sequential ultracentrifugation from sera of normolipidemic donors as previously described [9]. EDTA (0.40 g l^{-1}) was added to the sera to prevent spontaneous lipid peroxidation. The purity of LDLs was checked by agarose gel electrophoresis and by determining chemical composition. Concentrations of lipid components (total cholesterol, triacylglycerol, phospholipids) were assessed by enzymatic methods in a Olympus AU800

*Corresponding author. Fax: (33) (1) 53 73 97 08.

Abbreviations: CD, conjugated dienes; CE, cholesteryl ester; CEOOH, cholesteryl ester hydroperoxide; 5,6β-epoxy, 5,6β-epoxycholesterol; 7-OH, 7β-hydroxycholesterol; HPETE, hydroperoxyeicosatetraenoic acid; EDTA, ethylene diamine tetraacetic acid; HPLC, high performance liquid chromatography; 7-KC, 7-ketocholesterol; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substances

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analyzer (Olympus Diagnostica GmbH, Hamburg, Germany). Unesterified cholesterol was assayed using a kit from Biotrol (Paris, France). Total protein concentration was measured by the Lowry [20] technique using bovine serum albumin as standard.

2.2. Oxidative modifications of LDLs

2.2.1. Copper oxidation. After adjustment of LDL concentration at 1.50 g l^{-1} (expressed as total LDL concentration), lipoprotein preparations were dialyzed against 100 volumes of 10 mmol l^{-1} sodium phosphate buffer pH 7, containing 150 mmol l^{-1} sodium chloride, for 18 h, at 4°C in the dark. Oxidation was initiated at 37°C by addition of cupric sulfate (5 μmol l^{-1} final concentration) and was stopped by adding an EDTA solution (20 μmol l^{-1} final concentration) and cooling in an ice bath.

2.2.2. Water γ-radiolysis oxidation. The same LDL solutions (1.50 g l^{-1} total LDLs) were used for the radiolysis oxidation. LDLs were first dialyzed against 100 volumes of 0.01 mol l^{-1} sodium phosphate buffer pH = 7 for 18 h at 4°C in the dark. LDL solutions were then irradiated using a cesium 137 γ-ray source (activity: 6000 Ci) with a dose rate of 0.20 Gy s^{-1} under aerated conditions. These experimental conditions led to a simultaneous production of $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals with respective radiation yields of 2.80×10^{-7} and 3.40×10^{-7} mol J^{-1} [21].

2.3. Conjugated diene (CD) measurement

CD formation was monitored by measuring the absorbance signal at 234 nm (baseline = initial absorbance at 234 nm). From the absorbance profile for each LDL solution, three characteristic points can be determined that describe the oxidative behavior of the LDL preparation. In the copper-induced oxidation, the first point (the lag phase) was defined as the intersection of the baseline with the tangent of the slope of the absorbance curve during the propagation phase. By analogy with this lag phase expressed in minutes, we could define in the γ-radiolysis procedure a lag dose expressed in Gy [9]. Second, the maximum CD concentration was defined as the end of the propagation phase. The third point was the termination phase and corresponded to a constant CD concentration. CD concentrations were calculated with an absorbance coefficient $\varepsilon = 27\,000$ mol^{-1} l cm^{-1} [22]. For the study of oxysterol formation, we chose two points which were comparable with regard to their CD level, namely 2 h for copper oxidation and 800 Gy for γ-radiolysis.

2.4. Thiobarbituric acid-reactive substance measurement

TBARS were measured by a spectrofluorimetric method derived from that of Yagi [23] using the malondialdehyde kit from Sobioda Society (Grenoble, France).

2.5. Oxysterol measurement

Oxysterol measurement was achieved using the procedure described by Brown et al. [24]. Briefly, LDL preparations (1.50 g l^{-1} total LDLs) were divided into two fractions (each containing at least 200 μg of protein). One of these fractions was saponified in order to determine the total oxysterols while the other one was not saponified and allowed to determine oxysterols from unesterified cholesterol (oxysterols from esterified cholesterol were obtained by difference). 19-Hydroxycholesterol (Sigma Chemicals Co., St. Louis, MO, USA) was used as internal standard. Briefly, after saponification and extraction with diethylether and hexane, the samples were evaporated to dryness under a nitrogen stream. Trimethylsilyl (TMS) ether derivatives of the oxysterols were prepared by adding Fluka II Silylating Mixture (Supelco Inc., Bellefonte, PA, USA). The TMS ether derivatives of oxysterols were separated by gas chromatography (Hewlett-Packard 5890 A capillary gas chromatography), using a fused carbon-silica column (30 m × 0.25 mm i.d.) coated with (5% phenyl) methylpolysiloxane (DB-SMS; film thickness 1 μm) (Supelco Inc., Bellefonte, PA, USA) and detection by flame ionization. The TMS ether derivatives were carried by helium as vector gas to the column (temperature gradient from 260°C to 300°C with a constant temperature increase of 2°C min^{-1} during 20 min, then with a constant temperature of 300°C during 30 min). Standards (7β-hydroxycholesterol, 7α-hydroxycholesterol, α- and β-epoxy-cholesterol, 7-ketocholesterol, 25-hydroxycholesterol) were provided by Sigma Chemical Co. (St. Louis, MO, USA) and treated under the same conditions as the samples. Under our experimental conditions, the limit of detection of oxysterols was approximately 5 ng.

2.6. Measurement of molecular species of cholesteryl ester hydroperoxides

Lipids were extracted with methanol/hexane (4/10 v/v) from aliquots of LDL solutions. The hexane layer (upper phase containing cholesteryl esters) and the methanol/water layer (lower phase containing phospholipids) were separated by centrifugation at 1500 × *g* for 5 min. The upper layer was evaporated to dryness under a nitrogen stream, and the dried residue was then dissolved in methanol and injected into the HPLC system. Cholesteryl ester separation was performed as previously described [27] with a 250 × 4.60 mm C18 Spherisorb column. The mobile phase was methanol. Molecular species of CE were detected at 205 nm and the eluate was then mixed with the chemiluminescence reagent prepared as described by Yamamoto et al. [25] with slight modifications validated earlier by Thérond et al. [26]. Hydroperoxides from each molecular species of CE (linoleate and arachidonate, that is the two main species in LDLs) were prepared using lipoxygenase (type I-B, Sigma, St. Louis, MO, USA) as previously described [27]. Hydroperoxides from CE were extracted with methanol/hexane as described above for LDLs. CE hydroperoxides were detected by chemiluminescence (limit of detection = 30 pmol) and identified by their retention times [27].

2.7. Statistical analysis

Unless otherwise specified, results are presented as means ± S.D. of three experiments. Statistical analysis was carried out by Mann-Whitney's test (Apple Macintosh computer, Statview software). Values of $P < 0.05$ were considered significant.

3. Results

3.1. CD formation in γ-radiolysis- and copper-oxidized LDLs

Fig. 1 shows CD formation in 1.50 g l^{-1} LDLs (expressed as total LDLs) oxidized either by $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals (a) or by copper (5 μmol l^{-1} $CuSO_4$ final concentration) (b). As described in the literature [9,28], both curves exhibited three phases: a lag phase, a propagation phase and a termination phase. By analogy with what is classically observed in the copper-induced oxidation where the lag phase is the time which precedes the onset of the lipid chain peroxidation [28], we could define a lag dose as the radiation dose required for the onset of the lipid peroxidation process in the γ-radiolysis system [9]. The lag dose was equivalent to 70 ± 5 Gy (1 Gy = 1 J kg^{-1}) for the γ-radiolysis and the lag time was 45 ± 4 min for copper oxidation. The propagation rate, calculated as the slope of the linear part of the curve, was 0.40 ± 0.04 nmol mg^{-1} LDL Gy^{-1} for γ-radiolysis and 1.10 ± 0.10 nmol mg^{-1} LDL min^{-1} for copper oxidation. The major discrepancy between the two oxidation methods was the maximal level of CD formed, since it was higher after copper oxidation than after γ-radiolysis. A similar level of CD in LDLs was found after a radiation dose of 800 Gy and after 2 h of copper oxidation.

3.2. TBARS formation in γ-radiolysis- and copper-oxidized LDL

Fig. 1 shows the concentrations of TBARS in LDLs as a function of the radiation dose in γ-radiolysis oxidation (a) and of the oxidation time in copper oxidation (b). The TBARS concentrations obtained after γ-radiolysis increased as a function of the radiation dose until 800 Gy. In the copper oxidation system, the TBARS concentrations were of the same order of magnitude as those observed after γ-radiolysis. As previously observed for CD, LDLs exhibited a similar TBARS level after a radiation dose of 800 Gy and after 2 h of copper oxidation.

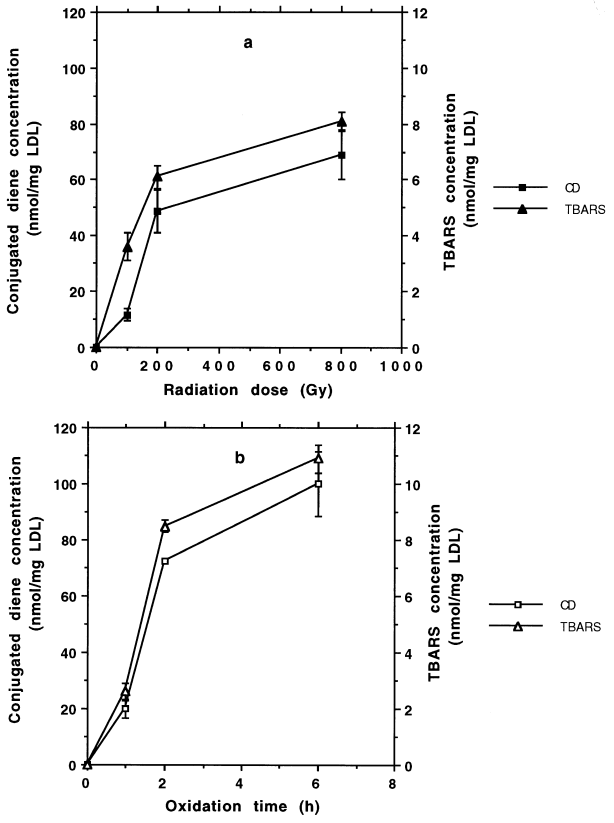

Fig. 1. CD and TBARS concentration in LDLs as a function of the radiation dose for γ-radiolysis (a) and as a function of the time of copper oxidation (b). a: Isolated LDLs (1.5 g l^{-1} total LDL) were irradiated in a sodium phosphate buffer (10 mmol l^{-1}, pH = 7) using a cesium 137 γ-ray source (activity: 6000 Ci) with a dose rate of 0.2 Gy s^{-1} under aerated conditions. Differential absorbance at 234 nm (reference = non-oxidized LDL, l = 1 mm) and TBARS concentration were determined after 100, 200, and 800 Gy irradiation. CD concentrations were calculated from measured absorbances using the absorbance molar coefficient ε = 27 000 mol^{-1} 1 cm^{-1}. b: The same LDLs (1.5 g l^{-1} expressed as total LDL concentration) were oxidized in a sodium phosphate (10 mmol l^{-1}, pH = 7) containing sodium chloride (150 mmol l^{-1}) at 37°C by addition of cupric sulfate (5 μmol l^{-1} final concentration). Differential absorbance at 234 nm (reference = non-oxidized LDL, l = 1 mm) and TBARS concentration were determined after 1, 2, and 6 h of copper oxidation. CD concentrations were calculated as described above. Results are means ± S.D. of three experiments carried out on LDL from normolipidemic donors.

3.3. Esterified and unesterified oxysterol formation

Table 1 represents the formation of 7-KC, 5,6β-epoxy and 7β-OH from esterified or unesterified cholesterol moieties either after γ-radiolysis (100, 200, 800 Gy) or after copper oxidation (1, 2, 6 h) of 1.5 g l^{-1} LDLs. Before oxidation, no oxysterol was detectable in LDLs. In both systems, 7-KC was the major oxysterol produced as compared to the level of the two other oxysterols. The formation of esterified and unesterified 7-KC was markedly lower by γ-radiolysis than by copper oxidation. In addition, radiolysis oxidation seemed to generate higher concentrations of 7-KC in the unesterified than in the esterified moiety. In contrast, at any time of copper oxidation, the level of 7-KC was higher in the cholesteryl ester moiety than in the unesterified cholesterol moiety.

As previously noted, the two other oxysterols were formed in lower concentrations than the 7-kKC, as well after γ-radiolysis as after copper oxidation. 5,6β-Epoxy and 7β-OH concentrations were always higher in the esterified fraction than in the unesterified one, either after γ-radiolysis or after copper oxidation. 5,6β-Epoxy was formed in the esterified fraction after 1 h of copper oxidation. In the unesterified fraction, 5,6β-epoxy was only detectable after 2 and 6 h of copper oxidation. γ-Radiolysis led to the formation of 5,6β-epoxy only after 800 Gy and only in the esterified fraction. 7β-OH was generated in the cholesteryl ester moiety after 1, 2 and 6 h of copper oxidation, whereas no 7β-OH appeared in the unesterified fraction. γ-Radiolysis induced a very low production of 7β-OH in the cholesteryl ester moiety, whereas 7β-OH was undetectable in the unesterified fraction.

3.4. Oxidative modification of polyunsaturated fatty acids from cholesteryl esters, and lipid hydroperoxide formation

Fig. 2 shows the concentration of linoleic and arachidonic acids esterified as major components (> 90%) of cholesteryl esters (CE) polyunsaturated fatty acids (PUFA) in native and oxidized LDLs. Native LDLs contained 380.00 ± 10.00 nmol CE PUFA mg^{-1} LDL. The oxidative degradation of esterified PUFA in LDL CE, expressed as percentage of PUFA in native LDL CE, increased as a function of the radiation dose

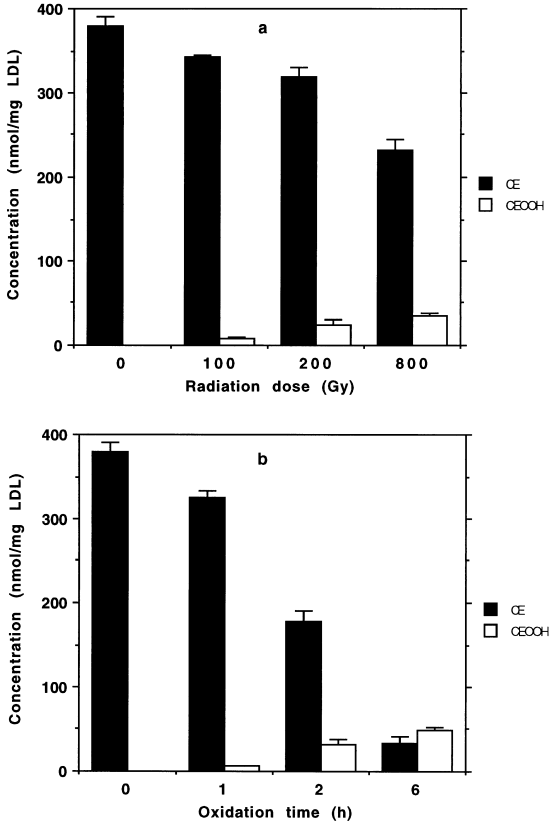

Fig. 2. Concentration of the cholesteryl ester (CE) polyunsaturated fatty acids (linoleic and arachidonic acids) (■) and cholesteryl ester hydroperoxides (CEOOH) (□) in LDL solutions (1.5 g l^{-1} total LDL) as a function of the radiation dose (a) and of the copper oxidation time (b). CEOOH species were isolated after a lipidic extraction from LDL solution (1.5 g l^{-1} total LDL). The hydroperoxide species of CE (from linoleic and arachidonic acids) were then separated by HPLC and measured by chemiluminescence as described in Section 2 at each radiation dose (100, 200, and 800 Gy) and at each time of copper oxidation (1, 2, and 6h).

Table 1
Esterified and unesterified 7-KC, 5,6β-epoxy and 7β-OH concentrations in LDL solutions (1.5 g l^{-1} total LDL) oxidized either by •OH and $O_2^{\bullet -}$ free radicals produced by γ-radiolysis (radiation dose: 100, 200, or 800 Gy) or by copper (oxidation time: 1, 2, or 6 h)

	•OH/$O_2^{\bullet -}$			5 μmol l^{-1} $CuSO_4$		
	100 Gy	200 Gy	800 Gy	1 h	2 h	6 h
Esterified						
7-KC	0.42 ± 0.10	0.94 ± 0.10	1.70 ± 0.30	7.90 ± 1.90	14.70 ± 3.30	34.90 ± 5.70
5,6β-Epoxy	ND	ND	1.40 ± 0.10	0.50 ± 0.10	1.50 ± 0.40	2.60 ± 0.40
7β-OH	0.30 ± 0.03	0.60 ± 0.03	1.80 ± 0.10	2.00 ± 0.20	5.80 ± 1.50	29.00 ± 7.40
Unesterified						
7-KC	0.74 ± 0.06	2.40 ± 0.20	2.90 ± 0.20	5.20 ± 0.70	7.60 ± 1.30	21.30 ± 3.20
5,6β-epoxy	ND	ND	ND	ND	0.20 ± 0.06	0.60 ± 0.02
7β-OH	ND	ND	ND	ND	ND	ND

ND: non-detectable.

(a). Copper oxidation (b) led to a CE PUFA consumption which was higher than that observed after γ-radiolysis. For example, it was approximately 2.5-fold higher after 6 h of copper oxidation than after a radiation dose of 800 Gy. In parallel with the oxidative modification of PUFA-containing CE, we monitored the formation of hydroperoxide species from polyunsaturated CE as a function of the oxidation. No hydroperoxide was detectable in native LDLs. Oxidation of LDLs led to an increase in CEOOH concentration as a function of the radiation dose after γ-radiolysis (a) and as a function of the oxidation time in the copper-induced oxidation procedure (b).

3.5. *Comparison between γ-radiolysis and copper oxidation at a similar level of lipid peroxidation*

In order to compare these two oxidation systems with regard to the oxysterol formation, we chose a radiation dose and an oxidation time corresponding to a similar level of lipid peroxidation, as assessed by the concentrations of CD and TBARS in oxidized LDLs (Table 2). This similar level was found for 800 Gy for γ-radiolysis and 2 h of copper oxidation. It is noteworthy that endogenous vitamin E (major LDL antioxidant) was totally consumed as well after a radiation dose of 800 Gy as after 2 h of copper oxidation (data not shown).

After 800 Gy irradiation, the esterified and unesterified 7-KC concentrations were very low. In contrast, after 2 h of copper oxidation, the 7-KC concentrations were markedly higher than after radiolysis (8.6-fold higher in the esterified moiety and 2.6-fold higher in the non-esterified one). This 7-KC concentration was 1.9-fold higher in the esterified than in the unesterified cholesterol moiety, which was in total contrast to what was observed after γ-radiolysis. Indeed, •OH and $O_2^{\bullet -}$ free radicals led after 800 Gy to a 1.7-fold lower concentration of 7-KC in the esterified moiety than in the unesterified fraction. The two other oxysterols assayed (namely 5,6β-epoxy and 7β-OH) were essentially found in the esterified moiety, as well after γ-radiolysis as after copper oxidation. It is noteworthy that •OH and $O_2^{\bullet -}$ free radicals led to equimolar con-

Table 2
Comparison between 1.5 g l^{-1} LDL oxidized either by •OH and $O_2^{\bullet -}$ free radicals produced by γ-radiolysis or by copper oxidation, at a similar level of lipid peroxidation

	•OH/$O_2^{\bullet -}$ (radiation dose: 800 Gy)	5 μmol l^{-1} $CuSO_4$ (oxidation time: 2 h)
CD concentration (nmol mg^{-1} LDL)	69.00 ± 8.60	73.00 ± 1.10^{NS}
TBARS concentration (nmol mg^{-1} LDL)	8.10 ± 0.30	8.50 ± 0.20^{NS}
Oxysterol concentration (nmol mg^{-1} LDL)		
Esterified		
7-KC	1.70 ± 0.30	14.70 ± 3.30*
5,6β-Epoxy	1.40 ± 0.10	1.50 ± 0.40^{NS}
7β-OH	1.80 ± 0.10	5.80 ± 1.50*
Unesterified		
7-KC	2.40 ± 0.20	7.60 ± 1.30*
5,6β-Epoxy	ND	0.20 ± 0.06
7β-OH	ND	ND
Total		
7-KC	4.60 ± 0.50	22.30 ± 4.60*
5,6β-Epoxy	1.40 ± 0.10	1.70 ± 0.46^{NS}
7β-OH	1.80 ± 0.10	5.80 ± 1.50*
Percent proportion of oxysterols derived from esterified and non-esterified cholesterol (%)		
Esterified		
7-KC	0.48	3.00
5,6β-Epoxy	0.40	0.40
7β-OH	0.51	1.60
Unesterified		
7-KC	1.10	3.00
5,6β-Epoxy	ND	0.08
7β-OH	ND	ND
CE PUFA consumption (nmol mg^{-1} LDL)	146.00 ± 14.00	200.20 ± 14.20*
CEOOH concentration (nmol mg^{-1} LDL)	34.20 ± 3.20	31.60 ± 2.40^{NS}

ND: non-detectable.
Differences between the two oxidation procedures: ^{NS}non-significant, $*P < 0.05$.

centrations of 7-KC and 7β-OH in the esterified fraction, which was not observed after 2 h of copper oxidation (7-KC concentration 2.5-fold higher than 7β-OH concentration).

Moreover, γ-radiolysis led to a lower consumption of CE PUFA than did copper oxidation. However, the concentration of CEOOH observed in the first case was slightly higher than after 2 h of copper oxidation.

4. Discussion

The aim of our study was to compare the formation of oxysterols in LDLs oxidized either by copper ions or by $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals produced by water γ-radiolysis. The general features of conjugated diene formation that we observed with the three typical phases (lag phase, propagation phase and termination phase) agreed with the literature data, as well with regard to γ-radiolysis oxidation [9] as with regard to copper oxidation [29]. Some of the biological properties of oxidized LDLs, especially cytotoxicity, are believed to be tightly dependent on LDL oxysterol content. As we compared two oxidation procedures which involved different mechanisms, it seemed of importance to be at a similar level of lipid peroxidation assessed by the concentration of conjugated dienes and TBARS in the oxidized LDLs. Our main results showed a markedly lower production of oxysterols (7-KC, 5,6β-epoxy and 7β-OH) after a radiation dose of 800 Gy than after 2 h oxidation by 5 μmol l^{-1} $CuSO_4$. This could be tentatively explained by two hypotheses: the first one would be a poor ability of $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals to produce oxysterols in LDLs, the second one would be supported by an apparent higher stability of the cholesteryl ester hydroperoxides in γ-radiolysis-oxidized LDLs, due to the absence of degradation of these hydroperoxides with copper ions.

In the present study, the formation of oxysterols was monitored during the course of LDL peroxidation induced by γ-radiolysis or copper. As well in the esterified as in the unesterified fraction, a markedly lower production of oxysterols was observed after action of $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals than after copper oxidation. As previously pointed out by Rice-Evans et al. [30], when LDLs are oxidized by two different procedures to the same extent as regards two markers of oxidation (i.e. conjugated dienes and TBARS), it does not follow that other oxidation products are present in similar amounts in these LDLs. No data were available in the literature on the formation of oxysterols in LDLs oxidized by $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals generated by γ-radiolysis. In contrast, formation of oxysterols has been largely described during copper- or cell-induced oxidation of LDLs in vitro, concomitantly with the loss of free and esterified cholesterol [24]. In agreement with others [24,29,31,32], we found that 7-KC was the major oxysterol formed during copper oxidation of LDLs. The kinetics of 7-KC formation as a function of the copper oxidation time and the percent proportion of oxysterols derived from esterified and unesterified cholesterol were in agreement with the data of Brown et al. [24]. Moreover, as previously shown in LDL copper oxidation [24], the three oxysterols assayed were essentially found in the esterified fraction of our copper-oxidized LDLs. This was likely related to the proportion of unesterified and esterified cholesterol molecules in LDLs, since an LDL particle contains about 600 molecules of unesterified cholesterol and 1600 molecules of esterified cholesterol [28].

By contrast, $^{\bullet}OH$ and $O_2^{\bullet -}$ free radicals led to a major formation of 7-KC in the unesterified fraction. Given the structure of an LDL particle [33] with the location of cholesteryl esters in the central core and the unesterified cholesterol at the LDL surface, it could be hypothesized that $^{\bullet}OH$ free radicals, which are very reactive and not selective [34], preferentially reacted with cholesterol molecules at the surface of LDL particles (that is, unesterified cholesterol), given the greater accessibility of these molecules. This would lead to a formation of oxysterols essentially from the unesterified fraction. This would not be the case for the copper oxidation which could perhaps preferentially involve secondary peroxyl radicals resulting from the oxidative attack of polyunsaturated fatty acids and reacting more deeply in the LDL particle.

Moreover, it is interesting to note that esterified 7-KC and 7β-OH were produced in equimolecular concentrations with the γ-radiolysis procedure at a similar level of lipid peroxidation (Table 2). This observation is very close to the mechanism recently proposed by Chang et al. [35] to explain cholesterol oxidation. According to these authors, cholesterol oxidation would lead to a 7-peroxyl radical of cholesterol which could disproportionate to give rise to one equivalent of 7β-OH, one equivalent of 7-KC and one dioxygen molecule, by means of a bimolecular decomposition reaction, as previously proposed by Smith [36]. However, such an equimolecular formation of 7-KC and 7β-OH was not obtained in our model of LDL copper oxidation, since approximately 2.5-fold more 7-KC than 7β-OH was formed after 2 h oxidation in the esterified fraction.

This led us to hypothesize that oxysterols could be secondarily formed from the decomposition of cholesteryl ester hydroperoxides (CEOOH).

Indeed, with regard to the kinetics of CEOOH formation upon copper oxidation, our data were of the same order of magnitude as the results published by Brown et al. [24,37] under similar experimental conditions. It is noteworthy that, under our conditions, the concentration of CEOOH formed after 2 h of copper oxidation (31.60 ± 2.40 nmol mg^{-1} LDL) was very low in comparison with the cholesteryl ester consumption (200.20 ± 14.20 nmol mg^{-1} LDL). This observation strongly suggested a secondary degradation of CEOOH, by reaction with copper ions, as previously described by Patel et al. [38] and by Ziouzenkova et al. [39]. Briefly, Cu^{2+} could be reduced to Cu^{+} by interaction with two reduction sites located in the apolipoprotein B of LDLs, then Cu^{+} and Cu^{2+} could react with CEOOH, leading to the respective formation of alkoxyl ($CEO^{\bullet}$) and peroxyl ($CEO_2^{\bullet}$) radicals. The direct attack of alkoxyl and peroxyl radicals on the C_7 of the cholesteryl esters could result in the formation of 7-ketocholesteryl esters [35]. The copper-mediated mechanism could not be proposed in the γ-radiolysis oxidation of LDLs, since transition metals were not involved. The results obtained after a radiation dose of 800 Gy (that is, at a similar lipid peroxidation level as after 2 h of copper oxidation) suggested a higher stability of the CEOOH generated than after copper oxidation. Indeed, the cholesteryl ester consumption was lower after γ-radiolysis (800 Gy) than after 2 h of copper oxidation (146.00 ± 14.00 vs. 200.20 ± 14.20 nmol mg^{-1} LDL), whereas CEOOH concentration was similar in both oxidation procedures.

In this study, the formation of oxysterols and molecular

species of CEOOH in LDLs oxidized by defined oxygen free radicals ($^{\bullet}OH/O_2^{\bullet-}$) was for the first time compared to that obtained with the commonly used copper oxidation procedure. The results clearly showed a markedly lower production of oxysterols induced by $^{\bullet}OH/O_2^{\bullet-}$ free radicals than by copper. This suggested a poor ability of these oxygen free radicals to induce cholesterol oxidation in LDLs. Moreover, the CEOOH produced by $^{\bullet}OH/O_2^{\bullet-}$ free radicals exhibited a higher stability than those formed upon copper oxidation, probably due to the absence of transition metals in the γ-radiolysis procedure. It is of interest to note that oxidized LDLs, even at a similar level of lipid peroxidation assessed by the concentration of conjugated dienes or TBARS, could exhibit major quantitative and/or qualitative differences with regard to their oxysterol content. Since oxysterols are highly involved in several biological effects of oxidized LDLs, especially cytotoxicity, γ-radiolysis allowed us to obtain convenient models of oxidized lipoproteins in order to get further insight into the understanding of their biological properties.

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