ELSEVIER

European Journal of Pharmacology 406 (2000) 429–437

ejp

www.elsevier.nl/locate/ejphar

Co-incubation of native and oxidized low-density lipoproteins: potentiation of relaxation impairment

Laurence Perségol *, Valérie Sementa, Anne Athias, Jean Lecerf, Christian Lallemant, Philippe Gambert

Unité INSERM U498, Métabolisme des Lipoprotéines Humaines et Interactions Vasculaires, Faculté de Médecine, Boulevard Jeanne d'Arc, BP 87900, 21079 Dijon Cedex, France

Received 21 June 2000; received in revised form 4 September 2000; accepted 5 September 2000

Abstract

The influence of native low-density lipoprotein (LDL) on the inhibition of endothelium-dependent relaxation previously induced by oxidized LDL was investigated with intact rabbit aortic rings. We also tried to assess oxysterol involvement in the native lipoprotein effects. Lipoprotein fractions (1 mg protein/ml) were tested for their ability to inhibit the vasorelaxation induced by acetylcholine in aorta rings previously precontracted by noradrenaline vs. that in control strips in Krebs buffer. Co-incubation of oxidized and native LDL reinforced the oxidized LDL-induced inhibition, compared to the impairment evoked by oxidized LDL alone ($E_{max} = 43.3 \pm 6.7\%$ and $61.4 \pm 5.4\%$, respectively; $P < 0.05$). Finally, smaller amounts of 7-oxy-cholesterols were recovered in organ baths after co-incubation of native and oxidized LDL than after incubation of oxidized LDL alone. Conversely, more oxy-c1holesterols were found in the strip vessels under the same conditions (% of oxysterol incorporation: 0.05158 vs. 0.10199, $r = 0.703$). Together these results suggest that the strengthening of oxidized LDL-induced inhibition by native LDL is dependent on an oxysterol effect on arterial wall cells. Mechanisms involved in this phenomenon remain to be investigated.

Keywords: Endothelium-dependent relaxation; Very low-density lipoprotein; Low-density lipoprotein; High-density lipoprotein; Oxysterol

1. Introduction

It is now well established that endothelium-dependent relaxation is impaired in atherosclerotic arteries. The impairment of vasoreactivity predisposes coronary arteries to vasospasm. The oxidized form of low-density lipoproteins (oxidized LDL) is involved in numerous atherogenic actions (Tanner et al., 1991; Kugiyama et al., 1990; Galle et al., 1991; Steinberg et al., 1989; Young and Parthasarathy, 1994) and its presence in atherosclerotic arteries has been demonstrated (Ylä-Herttuala et al., 1989). Even if native LDL has undefined effects, it is known that oxidized LDL induce a decrease of endothelium-dependent relaxation (Andrews et al., 1987; Tomita et al., 1990; Simon et al., 1990; Tanner et al., 1991; Lewis et al., 1997). Oxidation of LDL is associated with a decrease of compounds like cholesterol, phospholipids or antioxidants and with an increase of cholesterol oxides, lysophospholipids or lipoperoxides. Whereas experiments demonstrated that lysophosphatidylcholine does not mimic the inhibitory effect of oxidized LDL in porcine coronary arteries (Tanner et al., 1991), there are differences in results among studies (Mangin et al., 1993; Kugiyama et al., 1990). However, cholesterol oxides, especially derivatives in position 7, are directly connected with the inhibition of endothelium-dependent relaxation (Deckert et al., 1997).

High-density lipoproteins (HDL) play an important protective role in atherogenesis by promoting cholesterol reverse transport (Glomset, 1968; Pieters et al., 1994). They also protect LDL from oxidation (Mackness et al., 1993) and moreover, it has been proposed (Matsuda et al., 1993) that the inhibition of relaxation induced by oxidized LDL could be reversed by HDL by removal of lysophosphatidylcholine from oxidized LDL, thus preventing it from

* Corresponding author. Tel.: +33-3-80-39-32-62; fax: +33-3-80-39-32-64.

E-mail address: persegol@alcyone.u-bourgogne.fr (L. Perségol).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00696-8

acting on the endothelium. Consistent studies (Mackness et al., 1993; Watson et al., 1995) suggested that the HDL role was mediated via enzymatic processes such as paraoxonase. But nothing is known of the effects of co-incubation of native lipoprotein fractions such as LDL and very low-density lipoprotein (VLDL) with oxidized LDL on endothelium-dependent relaxation.

The aim of the present study was to determine the role of different native lipoprotein fractions in the inhibitory effect of oxidized LDL. Therefore, lipoproteins (VLDL, LDL and HDL) were isolated from several normolipidemic human plasmas and were kept native whereas other LDL were oxidized. We tested their ability to eliminate the inhibition of acetylcholine-mediated endothelium-dependent relaxation on rings pre-incubated with oxidized LDL. Moreover, we investigated whether native lipoprotein fractions could prevent the potent inhibitory effect of oxidized derivatives in position 7 of cholesterol on endothelium-dependent relaxation. Finally, we assessed, especially regarding co-incubation of native and oxidized LDL, the amounts of 7-oxy-cholesterol present in the organ baths and in the vessel wall to determine the part they may play in the co-incubation of native and oxidized LDL.

2. Materials and methods

2.1. Chemical compounds

Acetylcholine, 5-cholestene-3β-ol-7-one (7-keto-cholesterol), noradrenaline, sodium nitroprusside, fatty acid-free bovine serum albumin and butylated hydroxy-toluene were purchased from Sigma. As indicated by the supplier, cholesterol oxides were at least 98% pure. We confirmed the high degree of purity by capillary gas chromatography of cholesterol oxides.

2.2. Preparation of lipoproteins

Lipoproteins were prepared by sequential ultracentrifugation from fresh citrated plasma drawn from normolipidemic donors (Havel et al., 1955). Informed consent was obtained from donors. Densities were adjusted by addition of KBr. The different fractions were prepared from 300 ml of plasma in a 70.Ti rotor on an XL-90 ultracentrifuge (Beckman). The $d < 1.006$ g/ml fraction was obtained after an 8-h 50,000 rpm run. LDL ($1.019 < d < 1.063$ g/ml) were isolated after a 15-h 45,000 rpm run at the lowest density and a 24-h 45,000 rpm run at the highest density. Finally, HDL ($1.063 < d < 1.21$ g/ml) were prepared by a 40-h 50,000-rpm run at 1.21 g/ml density. All the different fractions were dialysed overnight against a 10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4 buffer (TBS buffer).

2.3. Oxidative modification of LDL

Oxidative modification of LDL was performed, according to Esterbauer et al. (1989), by incubation of freshly prepared LDL adjusted to a final concentration of 1.2 g protein/l in TBS, with a copper sulfate solution (final concentration, 5 μmol/l) for 24 h at 37°C. At the end, oxidation was stopped by the addition of EDTA (final concentration, 200 μmol/l) and butylated hydroxy-toluene (final concentration, 20 μmol/l) and LDL were kept at 4°C. Similarly, the native lipoproteins (VLDL, LDL and HDL, 1 g protein/l) were supplemented with EDTA (final concentration, 200 μmol/l) and butylated hydroxy-toluene (final concentration, 20 μmol/l) at 4°C.

2.4. Electrophoretic analysis

Native and oxidized LDL electronegativity was determined by agarose gel electrophoresis (Paragon Lipokit, Beckman) as previously described (Sparks and Phillips, 1992). Briefly, migration was performed in a barbital buffer pH 8.6. After 45-min electrophoresis at 100 V, gels were fixed with ethanol/acetic acid/water (90:15:45; v/v/v), stained with Sudan Black (0.07%) and de-stained with ethanol (45%). Electronegativity was then assessed with a Bio-Rad GS-670 imaging densitometer, using Molecular Analyst Software (Lab Bio-Rad, Hercules, USA). The degree of oxidation was quantified by using as an index, the relative mobility of oxidized LDL compared with that of native LDL.

2.5. Assay of cholesterol oxides

Total lipids from aorta cells or medium baths were extracted with the method of Folch et al. (1957). The extract was saponified at 60°C for 60 min with potassium hydroxide (13.2 g/l), followed by esterification at 60°C for 60 min with boron trifluoride (BF_3)–methanol to give fatty acid ethyl esters. Cholesterol oxides were analyzed by capillary gas chromatography (Park and Addis, 1985) on a Hewlett-Packard 12.5-m long fused silica, crossed-linked methylsilicone column with a Hewlett-Packard 5890 gas chromatograph attached to a 5971A mass detector (Hewlett-Packard). Concentrations of cholesterol and cholesterol oxides were determined from the ratio of the peak area corresponding to one given molecule, to the peak area corresponding to the internal standard (epi-coprostanol).

2.6. Preparation of blood vessels

Thirty New-Zealand White rabbits of either sex weighing 2.8–3.4 kg were killed by an overdose of pentobarbital sodium via the marginal ear vein. The descending aorta

was rapidly removed and transferred into a Krebs solution (composition in mmol/l: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 2.5, EDTA 0.027, glucose 11 and $NaHCO_3$ 25) bubbled with 95% O_2–5% CO_2 gas. The aorta was cut into 3-mm rings and suspended horizontally between two wire hooks in 5-ml jacketed organ baths containing oxygenated Krebs solution maintained at 37°C. One hook was connected to a force transducer (model UF1, Pioden) and the other was fixed to the support. Changes in isometric tension were monitored continuously on a Mac Lab 8 system (ADInstruments). The resting tension of the rings was set to 2 g and was similar to the resting tension used in many previous studies with rabbit aortic rings (Jacobs et al., 1990; Yokoyama et al., 1990; Andrews et al., 1987). After a 30-min equilibration period, the contractile response to KCl (30 mmol/l) was first obtained to check the contractile response of the vascular smooth muscle cells. This response to KCl constantly ranged between 8 and 10 g with all the arterial segments used in the present study. After washout and equilibration, the aortic rings were contracted with 0.3 μmol/l noradrenaline, a concentration giving 75% of the maximal contraction evoked by KCl. The contractile response to noradrenaline constantly ranged between 6 and 9 g with all the arterial segments used in the present study, and as determined by analysis of variance (ANOVA), no significant differences in the initial contraction in response to noradrenaline were observed after the various incubation protocols. After precontraction with noradrenaline, the rings were relaxed by cumulative additions of acetylcholine in the 1 nmol/l to 0.01 mmol/l concentration range. After washout and a 30-min recovery period, aortic rings were incubated for 2 h with native lipoproteins, oxidized LDL, or cholesterol oxides. The final concentration of lipoproteins in the organ bath (native or oxidized) was constantly 1 g protein/l and the final concentration of cholesterol oxides was 60 μg/ml. Control segments were incubated in parallel in Krebs buffer. At the end of incubation period, aortic segments were again contracted with noradrenaline and progressively relaxed with acetylcholine or with sodium nitroprusside.

2.7. Preparation of lipid solutions

5-Cholestene-3β-ol-7-one (7-keto-cholesterol) was first dissolved in ethanol (20 μl) and then mixed in a 1:1 molar ratio with a solution of fatty acid-free bovine serum albumin dissolved in Krebs buffer (7-keto-cholesterol final concentration 60 μg/ml).

2.8. Chemical analysis

All chemical assays were performed on a COBAS-BIO centrifugal analyser (Roche). Proteins were assayed with bicinchoninic acid reagent (Pierce) according to the method of Smith et al. (1985).

2.9. Data analysis

The maximal relaxation (E_{max}) induced by either acetylcholine or sodium nitroprusside and expressed as a percentage of the contraction with noradrenaline (0.3 μmol/l), was determined from experimental data. pD_2 values, corresponding to the negative logarithm of the concentration required to produce a half-maximum relaxing effect (EC_{50}), were determined after fitting each curve to a sigmoidal equation form $Y = P_1 + P_2/[1 + e^{P_3(\log x - P_4)}]$ in which x is the agonist concentration; P_1, the lower plateau response; P_2, the range between the lower and the maximum plateau of the concentration–effect curve; P_3, a negative curvature index indicating the slope independently of the range; P_4, $\log EC_{50}$ (Deckert et al., 1994). E_{max} and pD_2 values obtained with arterial rings pretreated with either oxidized LDL, native lipoproteins or cholesterol oxides were compared with corresponding E_{max} and pD_2 values obtained with control rings incubated in parallel only with Krebs solution.

Data are expressed as means ± S.E. Statistical comparison of means was performed with an ANOVA. Values of $P < 0.05$ were taken as statistically significant.

3. Results

3.1. Effects of oxidation on the composition of LDL particles

It is well established that oxidation changes the composition of LDL, especially the decrease of lipid components and antioxidants, was associated with an increase in cholesterol oxides, lipoperoxides and lysophosphatidylcholine (data not shown). The cholesterol derivatives oxidized in position 7 were the major products from cholesterol oxidation. Therefore, we have reported in Table 1 the amounts of these derivatives in native and oxidized LDL. Table 1 shows that only oxysterol traces were recovered in native LDL whereas oxidized LDL had high levels of cholesterol oxides, mainly 7β-hydroxy- and 7-keto-cholesterol. Before each experiment, the oxidation level

Table 1
Measurement of oxidation-derived oxysterols in LDL particles

	Native LDL	Oxidized LDL
7-α-Hydroxy-cholesterol (mg/g LDL protein)	0.90 ± 0.83	2.34 ± 0.65
7-β-Hydroxy-cholesterol (mg/g LDL protein)	3.0 ± 0.48	55.70 ± 15.73
7-Keto-cholesterol (mg/g LDL protein)	4.55 ± 0.94	105.42 ± 32.70

LDL particles were either kept at 4°C (native LDL) or incubated for 24 h at 37°C in the presence of $CuSO_4$ (final concentration 5 μmol/l) (oxidized LDL). Cholesterol oxides were assayed by capillary gas chromatography ($n = 5$).

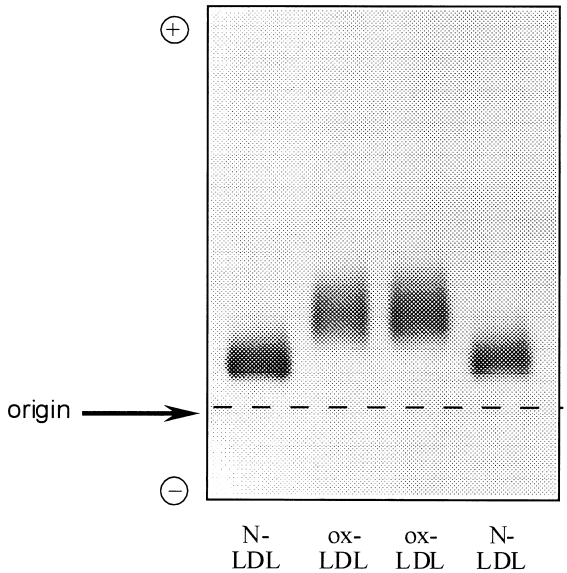

Fig. 1. Agarose gel electrophoresis of native and oxidized LDL. Electrophoresis was evaluated by scanning Bio-Rad with a GS-670. Each sample applied (5 μl) on agarose gel was run 45 min at 100 V. After separation, protein bands were visualized by Sudan Black staining.

was checked by measuring the change in electrophoretic mobility of oxidized LDL as shown in Fig. 1. The relative mobility of oxidized LDL (as index of oxidation) was 1.7 compared with that of native LDL.

3.2. Effects of oxidized LDL and native lipoproteins on endothelium-dependent relaxation

3.2.1. Effects of oxidized LDL alone

In accordance with results of previous experiments, oxidized LDL inhibited the endothelium-dependent relaxation to acetylcholine, compared with control segments (Fig. 2) (E_{max} 61.4 ± 5.4% vs. 96.9 ± 1.9%, respectively, $P < 0.001$). It is noteworthy that the difference was always observed although the inter-individual variations of aortas within the E_{max} were from 15.2 to 73.3 and pD_2 values ranged from 6.1 to 7.3 (data not shown).

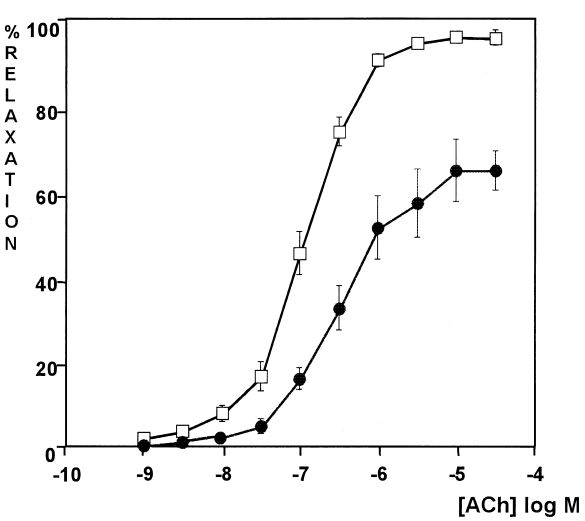

Fig. 2. Effect of oxidized LDL on endothelium-dependent relaxation of rabbit aorta induced by acetylcholine. Mean concentration–response curves for acetylcholine-induced relaxation of aorta rings contracted with 0.3 μmol/l noradrenaline and pre-incubated 2 h with either oxidized LDL (—●—) (1 g protein/l) or Krebs buffer (—□—) (control). Values represent means ± S.E. of 20 determinations.

3.2.2. Effects of native lipoproteins alone

When segments were pre-incubated with native VLDL, LDL or HDL (Fig. 3), the maximum relaxation with acetylcholine was slightly but significantly reduced by all the lipoproteins when compared with the relaxation of control aorta rings incubated in parallel in Krebs buffer (respectively 87.4 ± 7.1%, $P < 0.05$; 91.4 ± 3.0%, $P < 0.05$; and 91.6 ± 1.0%, $P < 0.01$ vs. 96.9 ± 1.9%). However, these changes were not associated with any changes in the sensitivity to acetylcholine since pD_2 values were not significantly affected (Table 2).

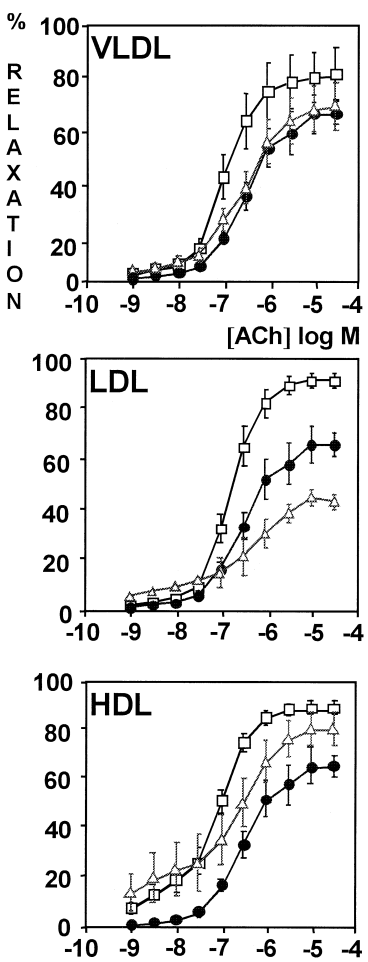

Fig. 3. Influence of oxidized LDL with or without native lipoproteins on the endothelium-dependent relaxation in rabbit aorta rings. Graphs show mean concentration–response curves for acetylcholine in aortas contracted with 0.3 μmol/l noradrenaline and pre-incubated 2 h with oxidized LDL (—●—) (1 g protein/l) either alone or with native VLDL, LDL and HDL (—△—) (1 g protein/l). Native lipoprotein (—□—). Values represent means ± S.E. of 4–20 determinations.

Table 2
Compared effects of the different native (n) lipoproteins with/without oxidized LDL or with/without 7-keto-cholesterol (7-keto) on maximal relaxation (E_{max}) and sensitivity (pD_2) of rabbit aortic rings to acetylcholine

	E_{max}	P	pD_2	P
Control	96.9 ± 1.9%		6.9 ± 0.1	
Ox-LDL	61.4 ± 5.4%	$P < 0.001$ (a)	6.5 ± 0.2	$P < 0.05$ (a)
n-VLDL	87.4 ± 7.1%	$P < 0.05$ (a)	6.9 ± 0.1	NS (a)
n-LDL	91.4 ± 3.0%	$P < 0.05$ (a)	6.7 ± 0.1	NS (a)
n-HDL	91.6 ± 1.0%	$P < 0.01$ (a)	7.2 ± 0.1	NS (a)
n-VLDL + ox-LDL	68.6 ± 9.0%	NS (b)	6.6 ± 0.2	$P < 0.05$ (b)
n-LDL + ox-LDL	43.3 ± 6.7%	$P < 0.05$ (b)	6.1 ± 0.3	$P < 0.001$ (b)
n-HDL + ox-LDL	80.9 ± 6.5%	$P < 0.05$ (b)	6.7 ± 0.5	NS (b)
7-Keto	74.5 ± 3.5%	$P < 0.001$ (a)	7.0 ± 0.1	NS (a)
n-VLDL + 7-keto	93.9 ± 0.8%	$P < 0.05$ (c)	7.0 ± 0.1	NS (c)
n-LDL + 7-keto	95.8 ± 1.7%	$P < 0.001$ (c)	6.9 ± 0.1	NS (c)
n-HDL + 7-keto	86.8 ± 2.9%	$P < 0.05$ (c)	7.2 ± 0.1	NS (c)

Aortic segments were incubated for 2 h with native lipoproteins with/without oxidized LDL or with/without 7-keto-cholesterol. The final concentration of 7-keto-cholesterol and proteins was respectively, 60 μg/ml and 1 g/l. Before and after the incubation period, a concentration–response curve to acetylcholine was made with noradrenaline-contracted aortas. Values represent means ± S.E. of 4–20 determinations. Statistical significance of the difference from control (a), from oxidized LDL (b) or from 7-keto-cholesterol (c) ANOVA.

3.2.3. Effects of native lipoproteins on the inhibition of relaxation induced by oxidized LDL

The effects of the different native lipoprotein fractions on oxidized LDL-induced inhibition of endothelium-dependent relaxation are presented in Fig. 3. As previously shown, co-incubation of native HDL (1 mg protein/ml) with oxidized LDL reduced the inhibitory effect of oxidized LDL on maximum relaxation when pre-incubated simultaneously (E_{max} HDL + oxidized LDL 80.9 ± 6.5% vs. oxidized LDL 61.4 ± 5.4% $P < 0.05$) (Table 2). However, as shown in Table 2, the reversal was not total and depended on the HDL dose (data not shown), moreover the sensitivity to acetylcholine was not significantly affected.

Native VLDL did not significantly influence the maximum inhibition induced by oxidized LDL (Table 2 and Fig. 3) (68.6 ± 9.0% vs. 61.4 ± 5.4%, respectively). However, they slightly decreased the sensitivity to acetylcholine since pD_2 were significantly lowered (6.6 ± 0.2 vs. control 6.9 ± 0.1, $P < 0.05$).

In contrast to HDL, native LDL reinforced the inhibitory effect of oxidized LDL on maximum relaxation (43.3 ± 6.7% vs. 61.4 ± 5.4%, $P < 0.05$) (Table 2). In addition, the pD_2 value of segments pre-incubated with LDL plus oxidized LDL were significantly lowered, com-

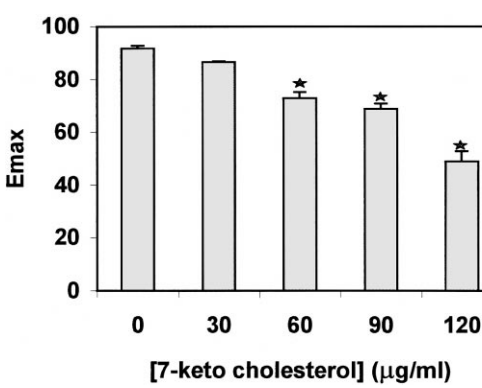

Fig. 4. Influence of 7-keto-cholesterol with or without native lipoproteins on the endothelium-dependent relaxation in rabbit aorta rings. Graphs show mean concentration–response curves for acetylcholine in aortas contracted with 0.3 μmol/l noradrenaline and pre-incubated 2 h with 7-keto-cholesterol (60 μg/ml) (—●—) alone, with/without native VLDL, LDL and HDL (— △ —) (1 mg protein/ml), native lipoprotein (— □ —). Values represent means ± S.E. of 4–20 determinations.

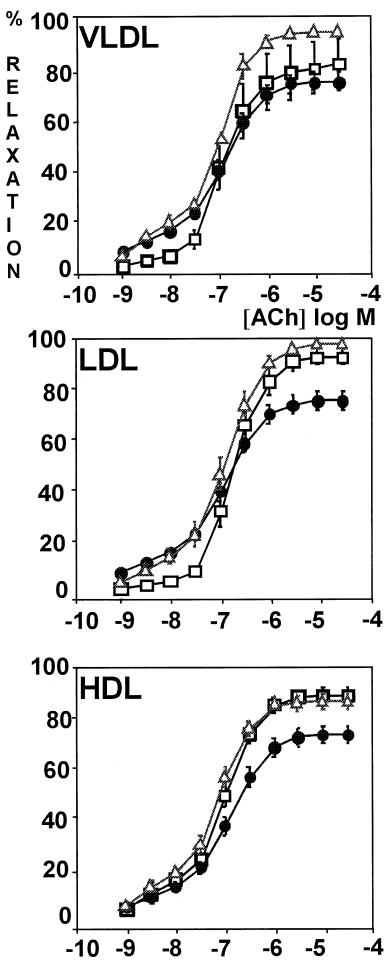

Fig. 5. Effect of 7-keto-cholesterol concentration on maximal endothelium-dependent relaxation. Aorta rings were incubated for 2 h with 7-keto-cholesterol, from 30 to 120 μg/ml, and effects were compared with those of aorta rings incubated in Krebs buffer. E_{max} histograms for acetylcholine were obtained after contraction with 0.3 μmol/l noradrenaline. Statistical significance compared with control (ANOVA): ★$P <$ 0.001.

pared to the control value (6.1 ± 0.3 vs. 6.9 ± 0.1, respectively, $P < 0.001$) but not significantly when compared to the oxidized LDL values (6.1 ± 0.3 vs. 6.5 ± 0.2, respectively). Native LDL pD_2 values, however, were not significantly modified (Table 2).

3.3. Effects of the various native lipoprotein fractions on the inhibition of the endothelium-dependent relaxation induced by 7-keto-cholesterol

Strips were pre-incubated with 7-keto-cholesterol (60 μmol/l final concentration) with/without the different native lipoprotein fractions. Compared to the control incubation in Krebs buffer, 7-keto-cholesterol inhibited endothelium-dependent relaxation (Fig. 4) in a dose-dependent manner (Fig. 5). At a final concentration of 60 μmol/l, it induced a significant inhibition of the endothelium-dependent relaxation ($74.5 \pm 3.5\%$ vs. $96.9 \pm 1.9\%$ for control bath, $P < 0.001$). When aorta rings were pre-incubated with 7-keto-cholesterol plus native lipoproteins, the inhibition induced by cholesterol oxides was always reversed by each native lipoprotein fraction (Fig. 5): the E_{max} of the rings in the presence of 7-keto-cholesterol plus VLDL, LDL and HDL were, respectively $93.9 \pm 0.8\%$ $P < 0.05$, $95.8 \pm 1.7\%$ $P < 0.001$ and $86.8 \pm 2.9\%$ $P < 0.05$ vs. $74.5 \pm 3.5\%$ for 7-keto-cholesterol alone (Table 2). No significant difference in pD_2 was observed.

3.4. Incubation of native lipoproteins with oxidized LDL: amount of oxidized derivatives in position 7 of cholesterol in the organ bath and in the vessel wall cells

Table 3 shows the assessment of 7-oxysterol amounts in the vessels and in the organ baths after a 2-h incubation. It appeared that, in three experiments, the rate of incorporation of the oxidized components was greater (0.09615, 0.13213 and 0.07769%, respectively, mean value: 0.10199) during the co-incubation of native and oxidized LDL, compared to incubation of oxidized LDL alone (0.03283, 0.07195 and 0.04996%, respectively, mean value: 0.05158) ($r = 0.703$). Moreover, the endothelium-dependent relaxation mediated by acetylcholine was correlated with the level of total 7-oxy-cholesterol incorporation in the wall cells ($r = -0.805$).

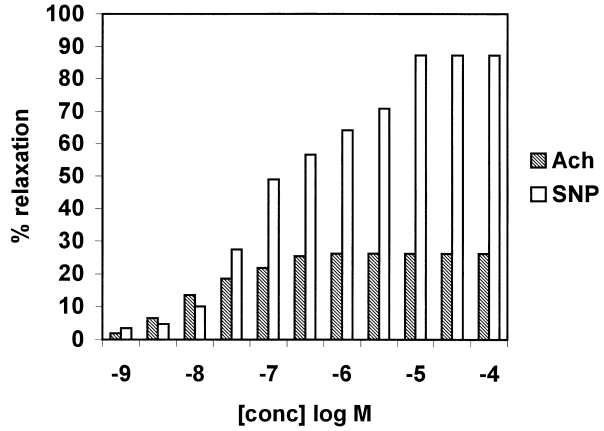

Fig. 6. Effect of co-incubation of native and oxidized LDL on independent and dependent endothelium relaxation induced respectively by sodium nitroprusside and acetylcholine. Graph shows concentration–response histograms for sodium nitroprusside (full bars) on aorta rings contracted with 0.3 μmol/l noradrenaline and incubated for 2 h with native and oxidized LDL compared with relaxation evoked by acetylcholine (hatched bars) in the same conditions. Each bar represents the mean of two experiments.

3.5. Comparison of native plus oxidized LDL co-incubation on aorta rings for endothelium-independent and -dependent relaxation

As shown in Fig. 6, co-incubation of native and oxidized LDL did not modify the endothelium-independent

Table 3
Measurement of total oxidized derivatives in position seven- of cholesterol in organ baths (1) and in arterial wall cells (2) after 2 h (T_2) of incubation with either particles or Krebs buffer (control) and consecutive endothelium-dependent relaxation after contraction with 0.3 μmol/l noradrenaline (3)

	(1) Total 7-OS amounts (μg) in organ-bath at T_2	(2) Total 7-OS amounts (ng) in aortic wall T_2	(3) % Relaxation	(4) % 7-OS incorporated in wall cells
Control no. 1	10.60	190	95.54	
Control no. 2	0.4	5.1	93.28	
Control no. 3	0.19	73	97.61	
Oxidized LDL no. 1	833.0	460	35.88	0.03283
Oxidized LDL no. 2	76.7	60	10.49	0.07195
Oxidized LDL no. 3	508.6	327	52.06	0.04996
Mean				**0.05158**
n-LDL + ox-LDL no. 1	405.65	570	5.94	0.09615
n-LDL + ox-LDL no. 2	86.6	119	0.45	0.13213
n-LDL + ox-LDL no. 3	452	424	7.84	0.07769
Mean				**0.10199**

Data obtained from three different experiments. The rate of incorporation of total 7-oxy-cholesterols (7-OS) was calculated from: (Total 7-OS in lipoproteins − Total 7-OS in control) in aortic wall cells/(Total 7-OS in lipoproteins-T − Total 7-OS in control) in organ baths. Cholesterol oxides were assayed by capillary gas chromatography.

relaxation when compared with the relaxation evoked by acetylcholine. In the two experiments presented mean values of E_{max} for sodium nitroprusside reached 87.2% whereas they only reached 26.2% for acetylcholine-induced relaxation.

4. Discussion

The major new finding from this study was that the inhibitory effect induced by oxidized LDL on endothelium-dependent relaxation, could be reinforced when aorta strips were incubated simultaneously with oxidized and native LDL. Moreover, this reinforcement is associated with oxysterol uptake by the arterial wall.

Many risk factors for the development of atherosclerosis have been identified and there is much evidence to indicate that oxidized LDL can lead to a decrease of endothelium-dependent relaxation through inhibition of production and/or release of endothelium-derived relaxing factor (EDRF-nitric oxide NO) (Galle et al., 1991). On rabbit aorta rings (Tanner et al., 1991; Jacobs et al., 1990; Kugiyama et al., 1990; Galle et al., 1991; Deckert et al., 1997), the inhibitory effects of oxidized LDL are mediated the oxidized lipids produced. In the current study, LDL were oxidized by using copper. The oxidized particles obtained in this case had chemical and biological properties similar to those of cell-modified LDL (Steinbrecher et al., 1984). It should be noted that the increase in electrophoretic anionic mobility, because of the more important net negative charge of oxidized LDL, was used to evaluate oxidation (Fig. 1).

The effects of the different native lipoprotein fractions on vasorelaxation are very controversial. Previous experiments have shown that all native lipoparticles could impair vasorelaxation (Andrews et al., 1987; Takahashi et al., 1990; Tomita et al., 1990; Jacobs et al., 1990; Galle et al., 1991; Lewis et al., 1997; Grieves et al., 1998). The differences between our results and those of others are probably due to oxidation possibly taking place in the organ bath. In our study, the lipoproteins were protected from oxidative processes by butylated hydroxy-toluene which is a free-radical scavenger, whereas EDTA is only a metal chelator and consequently only prevents metal-induced oxidation.

As in previous studies (Matsuda et al., 1993; Galle et al., 1994a,b), we verified that native HDL co-incubated with oxidized LDL in the organ bath, were protective for vessels by allowing relaxation, probably through removal of lysophosphatidyl choline from oxidized LDL (Matsuda et al., 1993) and/or through an enzymatic process, possibly paraoxonase (Mackness et al., 1993). Incubation of vessel rings with VLDL plus ox-LDL led to heterogenous E_{max} (from 10.2% to 84.6%, mean $68.6 \pm 9\%$). The wide range of the results could be explained by two phenomena. (i) In genetically hyperlipidemic rabbits, only small VLDL could be taken up and trapped in the inner arterial media intima. In this case, VLDL are at least as atherogenic as LDL (Nordestgaard et al., 1995). (ii) Moreover, Vogel et al. (1997) have shown that post-prandial hypertriglyceridaemia transiently impairs endothelium-dependent flow-mediated vasodilatation.

In contrast to native HDL, native LDL did not reverse the oxidized LDL-induced inhibition of endothelium-dependent vasodilatation: co-incubation of oxidized LDL plus native LDL induced a more potent inhibition of endothelium-dependent relaxation (Fig. 3).Through which pathways could native LDL reinforce the oxidized LDL induced-inhibition of endothelium-dependent vasorelaxation is the question.

Recently, cholesterol oxides, and more precisely, oxidized derivatives in position 7 of cholesterol, were demonstrated to be the main factor of oxidized LDL-induced inhibition of endothelium-dependent vasodilatation (Deckert et al., 1997). Moreover, cytotoxic properties associated with 7-keto-cholesterol and 7-hydroxy-cholesterol (Hughes et al., 1994) have been shown, particularly on endothelial cells (Lizard et al., 1996). The oxygenated derivatives of cholesterol can originate in different ways, one possibility being from LDL oxidation; oxidized derivatives in position 7 of cholesterol are the first oxysterols formed during LDL in vitro oxidation and found in human atherosclerotic femoral arteries (Patel et al., 1996; Björkhem et al., 1994; Carpenter et al., 1993).

To determine whether oxidized derivatives in position 7 of cholesterol could be the factors involved in reinforcement of vasorelaxation inhibition, two kinds of experiments were performed: first, co-incubation of 7-keto-cholesterol plus native lipoproteins to assess the influence on endothelium-dependent vasorelaxation and second, co-incubation of native particles with oxidized LDL to estimate the total oxidized derivatives in position 7 of cholesterol entry in the arterial wall.

As already reported (Deckert et al., 1997), 7-oxysterols inhibit endothelium-dependent relaxation (Fig. 4) in a dose-dependent manner (Fig. 5) compared with the control values. However, co-incubation of 7-keto-cholesterol with native lipoproteins, including LDL and VLDL, eliminated the inhibitory effect. It could be hypothesized that during incubation, 7-oxysterols are taken up by native lipoproteins and therefore could protect them from toxicity. Native LDL could reverse the inhibition induced by 7-keto-cholesterol (Fig. 4) but, on the contrary, reinforce the inhibition when they are incubated with oxidized LDL (Fig. 3). It can be supposed that native LDL could pick up oxysterols when they are linked to particles in the organ bath which are not lipoproteins (albumin, for example), but not when they are already present in oxidized LDL (Table 3).

Measurement of total 7-oxy-cholesterols in the organ bath and in the aorta rings at the end of incubation (Table 3) has shown that native LDL favour their uptake by the

inner part of the aorta ring. Moreover, their incorporation is increased when native LDL are incubated with oxidized LDL compared to the incorporation induced by oxidized LDL alone. It also appeared that the level of inhibition seems to be correlated with 7-oxysterol incorporation rate (Table 3). Our results are in good agreement with those obtained by Rong et al. (1998). They demonstrated that cholesterol oxidation products gradually increased after 70-day injection period, and accumulated in the inner half of the media of rabbit thoracic aorta. Moreover, neither 7-oxy-cholesterol incubation (Deckert et al., 1997), nor native plus oxidized lipoprotein co-incubation (Fig. 6) could affect the arterial relaxation mediated by sodium nitroprusside. These results indicate that the inhibition was due to endothelial mechanisms rather than to a direct effect on smooth muscle cells. It could be hypothesized that 7-oxy-cholesterol incorporated in the vessel wall cells could induce nitric oxide release or alter its production, a hypothesis which could be corroborated by the study of Deckert et al. (1998) which showed that 7-oxy-cholesterols could reduce the histamine-activated nitric oxide release in human umbilical vein endothelial cells.

In conclusion, the data now presented demonstrate that co-incubation of different native lipoprotein fractions with oxidized LDL produce opposite effects on atherogenesis. The especially interesting finding is that native LDL reinforces the inhibitor effect of oxidized LDL and therefore could play an atherogenic role. The strengthening effects of native LDL seem to be associated with cholesterol oxide uptake by the arterial wall and to their possible action on nitric oxide metabolism.

Acknowledgements

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Université de Bourgogne and the Conseil Régional de Bourgogne.

References

Andrews, H.E., Bruckdorfer, R.K., Dunn, R.C., Jacobs, M., 1987. Low-density lipoproteins inhibits endothelium-dependent relaxation in rabbit aorta. Nature 327, 237–239.

Björkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R.J., Duan, C., Lund, E., 1994. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. Proc. Natl. Acad. Sci. 91, 8592–8596.

Carpenter, K.L.H., Taylor, S.E., Ballantine, J.A., Fussell, B., Halliwell, B., Mitchinson, M.J., 1993. Lipids and oxidized lipids in human atheroma and normal aorta. Biochim. Biophys. Acta 1167, 121–130.

Deckert, V., Pruneau, D., El-Ghozi, J.L., 1994. Mediation by 5-HT1D receptors of 5-hydroxytryptamine-induced contractions of rabbit middle and posterior cerebral arteries. Br. J. Pharmacol. 112, 939–945.

Deckert, V., Perségol, L., Viens, L., Lizard, G., Athias, A., Lallement, C., Gambert, P., Lagrost, L., 1997. Inhibitors of arterial relaxation among components of human oxidized low-density lipoproteins. Circulation 95, 723–731.

Deckert, V., Brunet, A., Lantoine, F., Lizard, G., Millanvoye-van Brussel, E., Monier, S., Lagrost, L., David-Dufihlo, M., Gambert, P., Devynck, M.A., 1998. Inhibition by cholesterol oxides of NO release from human vascular endothelial cells. Arterioscler., Thromb., Vasc. Biol. 18, 1054–1060.

Esterbauer, H., Striegl, G., Puhl, H., Rotheneder, M., 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Radical Res. Commun. 6, 67–75.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Galle, J., Mülch, A., Busse, R., Bassenge, E., 1991. Effects of native and oxidized low-density lipoproteins on formation and inactivation of endothelium-derived relaxing factor. Arterioscl. Thromb. 11, 198–203.

Galle, J., Bengen, J., Schollmeyer, P., Wanner, C., 1994a. Oxidized lipoprotein(a) inhibits endothelium-dependent dilation: prevention by high-density lipoprotein. Eur. J. Pharmacol. 265, 111–115.

Galle, J., Öchslen, M., Schollmeyer, P., Wanner, C., 1994b. Oxidized lipoproteins inhibit endothelium-dependent vasodilatation. Hypertension 23, 556–564.

Glomset, J.A., 1968. The plasma lecithin: cholesterol acyltransferase. J. Lipid Res. 9, 155–167.

Grieves, D.J., Avella, M.A., Botham, K.M., Elliott, J., 1998. Effects of chylomicrons and chylomicron remnants on endothelium-dependent relaxation of rat aorta. Eur. J. Pharmacol. 348, 181–190.

Havel, R.J., Eder, H.A., Bragdon, J.H., 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34, 1345–1353.

Hughes, H., Mathews, B., Lenz, M.L., Guyton, J.R., 1994. Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells is associated with the oxysterols 7-keto-cholesterol and 7-hydroxycholesterol. Arterioscl. Thromb. 14, 1177–1185.

Jacobs, M., Plane, F., Bruckdorfer, R.K., 1990. Native and oxidized low-density lipoproteins have different inhibitory effects on endothelium-derived relaxing factor in the rabbit aorta. Br. J. Pharmacol. 100, 21–26.

Kugiyama, K., Kerns, S.A., Morrisett, J.D., Roberts, R., Henry, P.D., 1990. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low density liporoteins. Nature 344, 160–162.

Lewis, T.V., Dart, A.M., Chin-Dusting, J.P.F., 1997. Non-specific inhibition by human lipoproteins of endothelium-dependent relaxation in rat aorta may be attributed to lipoprotein phospholipids. Cardiovasc. Res. 34, 590–596.

Lizard, G., Deckert, V., Dubrez, L., Moisant, M., Gambert, P., Lagrost, L., 1996. Induction of apoptosis in endothelial cells treated with cholesterol oxides. Am. J. Pathol. 148, 16125–16138.

Mackness, M.I., Arrol, S., Abbott, C., Durrington, P.N., 1993. Protection of low-density lipoprotein against oxidative modification by high-density density lipoprotein associated paraoxonase. Atherosclerosis 104, 129–135.

Mangin, E.L., Kugiyama, K., Nguy, G.H., Kerns, S.A., Henry, P.D., 1993. Effects of lysolipids and oxidatively-modified low-density lipoprotein on endothelium-dependent relaxation of rabbit aorta. Circ. Res. 72, 161–166.

Matsuda, Y., Hirata, K.I., Inoue, N., Suematsu, M., Kawashima, S., Akita, H., Yokoyama, M., 1993. High-density lipoprotein reverses inhibitory effect of oxidized low-density lipoprotein on endothelium-dependent arterial relaxation. Circ. Res. 72, 1103–1109.

Nordestgaard, B.G., Wootton, R., Lewis, B., 1995. Selective retention of VLDL, IDL, and LDL in the arterial intima of genetically hyperlipidemic rabbits in vivo. Arterioscler., Thromb., Vasc. Biol. 15, 534–542.

Park, S.W., Addis, P.B., 1985. Capillary column gas–liquid chromatographic resolution of oxidized cholesterol derivatives. Anal. Biochem. 149, 274–283.

Patel, R.T., Diczfalusy, U., Dzeletovic, S., Wilson, M.T., Darley-Usmar, V.M., 1996. Formation of oxysterols during oxidation of low-density lipoprotein by peroxynitrite, myoglobin, and copper. J. Lipid Res. 37, 2361–2371.

Pieters, M.N., Schouten, D., van Berker, T.J.C., 1994. In vitro and in vivo evidence for the role of HDL in reverse cholesterol transport. Biochim. Biophys. Acta 1225, 125–134.

Rong, J.X., Rangaswamy, S., Shen, L., Dave, R., Chang, Y.H., Peterson, H., Hodis, H.N., Chisolm, G.M., Sevanian, A., 1998. Arterial injury by cholesterol oxidation products causes endothelium dysfunction and arterial wall cholesterol accumulation. Arterioscler., Thromb., Vasc. Biol. 18, 1885–1894.

Simon, B.C., Cunningham, L.D., Cohen, R.A., 1990. Oxidized low-density lipoproteins cause contraction and inhibit endothelium-dependent relaxation in the pig coronary artery. J. Clin. Invest. 86, 75–79.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzo, M.D., Fugimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Sparks, D.L., Phillips, M.C., 1992. Quantitative measurement of lipoprotein surface charge by agarose gel electrophoresis. J. Lipid Res. 33, 123–130.

Steinberg, D., Parthasarathy, S., Carew, T.E., Witzum, J.L., 1989. Beyond cholesterol: modification of low-density lipoprotein that increase its atherogeneicity. N. Engl. J. Med. 320, 915–924.

Steinbrecher, U.P., Parthasarathy, S., Leake, D., Wiztum, J.L., Steinberg, D., 1984. Modification of low-density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low-density lipoprotein phospholipids. Proc. Natl. Acad. Sci. U. S. A. 81, 3883–3887.

Takahashi, M., Yui, Y., Yasumoto, H., Aoyama, T., Morishita, H., Hattori, R., Kawai, C., 1990. Lipoproteins are inhibitors of endothelium-dependent relaxation of rabbit aorta. Am. J. Physiol. 258, H1–H8.

Tanner, F.C., Noll, G., Boulanger, C.M., Lüscher, T.F., 1991. Oxidized low-density lipoproteins inhibits relaxations of porcine coronary arteries: role of scavenger receptor and endothelium-derived NO. Circulation 83, 2012–2020.

Tomita, T., Ezaki, M., Miwa, M., Nakamura, K., Inoue, Y., 1990. Rapid and reversible inhibition by low-density lipoprotein of the endothelium-dependent relaxation to hemostatic substances in porcine coronary arteries: heat- and acid-labile factors in low-density lipoprotein mediate the inhibition. Circ. Res. 66, 18–27.

Vogel, R.A., Corretti, M.C., Plotnick, G.D., 1997. Effect of a single high-fat meal on endothelium function in healthy subjects. Am. J. Cardiol. 79, 350–354.

Watson, A.D., Berliner, J.A., Hama, S.Y., La Du, B.N., Faull, K.F., Fogelman, A.M., Navab, M., 1995. Protective effect of high-density lipoprotein associated paraoxonase. J. Clin. Invest. 96, 2882–2891.

Ylä-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witzum, J.L., Steinberg, D., 1989. Evidence for the presence of oxidatively modified low-density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest. 84, 1086–1095.

Yokoyama, M., Harita, K.I., Miyake, R., Akita, H., Ishikawa, Y., Fusaki, H., 1990. Lysophosphatidylcholine: essential role in the inhibition of endothelium-dependent relaxation by oxidized low-density lipoprotein. Biochem. Biophys. Res. Commun. 168, 301–308.

Young, S.G., Parthasarathy, S., 1994. Why are low-density lipoproteins atherogenic? West J. Med. 160, 153–164.