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Detection of new epitopes formed upon oxidation of low-density lipoprotein, lipoprotein (a) and very-low-density lipoprotein

Use of an antiserum against 4-hydroxynonenal-modified low-density lipoprotein

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4-Hydroxynonenal (HNE) is a major aldehydic propagation product formed during peroxidation of unsaturated fatty acids. The aldehyde was used to modify freshly prepared human low-density lipoprotein (LDL). A polyclonal antiserum was raised in the rabbit and absorbed with freshly prepared LDL. The antiserum did not react with human LDL, but reacted with $CuCl_2$-oxidized LDL and in a dose-dependent manner with LDL, modified with 1, 2 and 3 mM-HNE, in the double-diffusion analysis. LDL treated with 4 mM of hexanal or hepta-2,4-dienal or 4-hydroxyhexenal or malonaldehyde (4 or 20 mM) did not react with the antiserum. However, LDL modified with 4 mM-4-hydroxyoctenal showed a very weak reaction. Lipoprotein (a) and very-low-density lipoprotein were revealed for the first time to undergo oxidative modification initiated by $CuCl_2$. This was evidenced by the generation of lipid hydroperoxides and thiobarbituric acid-reactive substances, as well as by a marked increase in the electrophoretic mobility. After oxidation these two lipoproteins also reacted positively with the antiserum against HNE-modified LDL.

INTRODUCTION

The cells responsible for the formation of early atherosclerotic lesions, namely the macrophages, are normally unable to take up LDL to any great extent. However, *in vitro*, macrophages were shown to ingest large amounts of LDL that had been chemically modified by acetylation [1]. By this procedure the ϵ-amino group of the lysine residues of apolipoprotein B (apoB) of LDL becomes blocked, with the net negative charge of the lipoprotein being strongly enhanced. In 1981 it was demonstrated by Henriksen *et al.* [2] that incubation of LDL with endothelial cells also causes a modification of LDL. This modified LDL was recognized by the receptors for acetylated LDL on macrophages. In the reports by Morel *et al.* [3] and Steinbrecher *et al.* [4] which followed, it was shown that a free-radical oxidation (e.g. lipid peroxidation) is involved in the alteration of LDL by endothelial cells. Furthermore, the way in which LDL can be modified by cells can also be mimicked by incubation of the lipoprotein solely in the presence of micromolar concentrations of $CuCl_2$ [5]. Studies performed by Fogelman *et al.* [6] showed that incubation of LDL with malonaldehyde (MDA), one of the main decomposition products generated on peroxidation of arachidonic acid, led finally to cholesteryl ester accumulation in human monocytes/macrophages after a critical number of lysine residues had been modified by MDA [7]. These findings induced us to investigate whether and in which way 4-hydroxynonenal (HNE), another main product of lipid peroxidation, was capable of modifying LDL [8]. In a later report studying the kinetics of the oxidation of the lipids in LDL, it was demonstrated that HNE is in fact generated among a series of other aldehydic products in the lipid phase of LDL [9,10]. However, complete evidence was still lacking that HNE or any of the related aldehydes had created new epitopes on the surface after generation *in situ* in oxidized LDL. Thus, as a first step, an antiserum was raised in the rabbit against human LDL modified by HNE in order to investigate the epitopes newly formed upon oxidation of LDL. Additionally, we tested whether lipoprotein (a) [Lp(a)] and very-low-density lipoprotein (VLDL), also considered to be a risk factor for the development of atherosclerosis when elevated in the plasma, were susceptible to oxidation. Furthermore, we decided to find out whether their oxidized forms reacted positively with the antiserum.

MATERIALS AND METHODS

Lipoprotein preparation

Lipoproteins were isolated from plasma of normolipaemic, fasting (12–14 h), young male and female donors (< 25 years). Chloramphenicol (50 mg/litre; Serva, Heidelberg, Germany), kallikrein inactivator (Trasylol; 100000 units/litre; Bayer, Leverkusen, Germany), butylated hydroxytoluene (BHT; 20 μM; Sigma, St. Louis, MO, U.S.A.) and EDTA (1 g/litre; Merck, Darmstadt, Germany) were present during all steps of lipoprotein preparation to prevent lipid peroxidation and apoB cleavage by contaminating bacteria or proteinases. Only plasma from donors with an Lp(a) serum level below 1 mg/dl was used for LDL and VLDL preparations. By differential ultracentrifugation, using solid

Abbreviations used: HNE, 4-hydroxynonenal; LDL, low-density lipoprotein; Lp(a), lipoprotein (a); VLDL, very-low-density lipoprotein; apoB, apolipoprotein B; MDA, malonaldehyde; LPO, lipid peroxides; TBARS, thiobarbituric acid-reactive substances; BHT, butylated hydroxytoluene.

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KBr to adjust the density, the following fractions were obtained: LDL, 1.020–1.050 g/ml; VLDL, 0.950–1.006 g/ml. Lp(a) was isolated from one female donor with an LP(a) serum level of 50 mg/dl in the density range 1.050–1.110 g/ml as described elsewhere [11]. In order to obtain a complete separation of Lp(a) from contaminating LDL, the fraction obtained by ultracentrifugation was purified on Bio-Gel A 15 M (Bio-Rad Laboratories, Richmond, CA, U.S.A.) as reported previously [12].

Modification of the lipoproteins

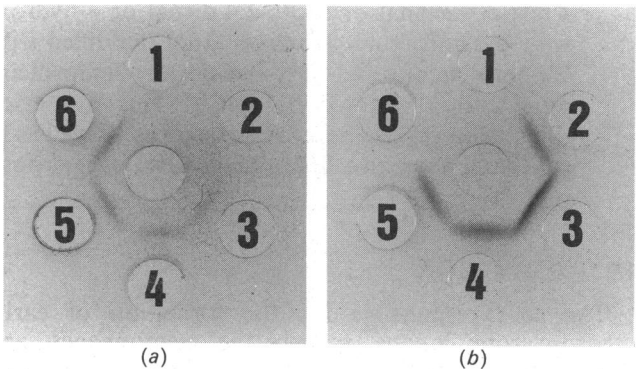
HNE, 4-hydroxyhexenal and 4-hydroxyoctenal were synthesized as described previously [13]. Aqueous solutions of these aldehydes were prepared as previously described for HNE [8], except that instead of the Tris/HCl buffer, 0.01 M-phosphate-buffered saline, pH 7.4, containing 1 g of EDTA/litre as well as 50 mg of chloramphenicol/litre and saturated with N_2, was used. MDA was obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane [14] and its content was measured as described in [15]. Hexanal and hepta-2,4-dienal were supplied by Aldrich, Steinheim, Germany. After having been dialysed against the above-mentioned buffer, suitable portions of LDL were incubated with appropriate amounts of the various aldehydes, producing a final concentration of the lipoprotein of 1.5 mg/ml. Incubations were performed in the dark at 37 °C for 5 h. Excess aldehyde was removed by dialysis against the buffer described above. Oxidation of the lipoproteins was performed using concentrations of 10 and 50 μM-$CuCl_2$. Before oxidation the lipoproteins were carefully dialysed against 0.01 M-phosphate-buffered saline, pH 7.4, containing chloramphenicol (50 mg/litre). The buffer was carefully degassed and saturated with N_2. Oxidation was performed using time intervals of 6 and 24 h. The reaction was terminated by adding a stop solution such that the final concentrations of BHT and EDTA in the lipoprotein solution were 20 μM and 24 μM respectively. Lipid peroxides (LPO) were evaluated by a method described recently [16]. Thiobarbituric acid-reactive substances (TBARS) were determined by using the method for the estimation of MDA [15] mentioned above. The electrophoretic runs were performed on agarose gels (1%) at pH 8.05 using the Lipidophor System (Immuno AG, Vienna, Austria).

Immunological procedures

The preparation of the antiserum against HNE-treated LDL was carried out using LDL from a single donor with a serum Lp(a) level below 1 mg/dl. LDL (1.5 mg/ml) was treated with 4 mM-HNE as described above. Rabbits were immunized by intracutaneous injections of the modified lipoprotein (500 μg of protein) emulsified in complete Freund's adjuvant. Two boosters at multiple sites were given at 4-week intervals. Antibodies not directed against the epitope created by HNE were precipitated by the addition of certain amounts of native LDL from the same donor. For instance, to 1 ml of antiserum 35 μl of LDL ($\equiv$ 0.7 mg total lipoprotein) was added and incubated for 2 h at 37 °C. The precipitate was removed by centrifugation at 6000 *g* in an Eppendorf centrifuge. This procedure was repeated twice. The specificity of the antiserum was assessed by double-diffusion analysis. Double-diffusion analysis was performed in 1% agarose (Agarose Standard Low-M_r; Bio-Rad Laboratories, Richmond, CA, U.S.A.). The antiserum was used without dilution, 16 μl being applied to the middle well. A 6 μg portion of lipoprotein protein was applied to each of the surrounding wells. Staining was performed with Amido Black.

RESULTS AND DISCUSSION

The polyclonal antiserum against HNE-treated LDL did not react with native LDL in the double-diffusion analysis as shown in Fig. 1(*a*). A positive reaction was obtained with LDL treated with 1, 2 and 3 mM-HNE. The relative electrophoretic mobility of HNE-treated LDL increased from 1 to 3 mM-HNE owing to an increase in modification with increasing amounts of HNE (Table 1). This was consistent with the strength of precipitation in the double-diffusion analysis (Fig. 1*a*). LDL modified by



(*a*) (*b*)

Fig. 1. Double-diffusion analysis of modified and oxidized LDL with the antiserum raised against LDL modified by HNE

The antiserum was applied to the middle well. A 6 μg portion of lipoprotein protein was applied to each well (1–6). (*a*) Well 1, control LDL; 2, LDL treated with 20 mM-MDA; 3, LDL treated with 1 mM-HNE; 4, 2 mM-HNE; 5, 3 mM-HNE; 6, LDL treated simultaneously with 4 mM-HNE and 20 mM-MDA. (*b*) Well 1, control LDL; 2, LDL modified with 3 mM-HNE; 3, LDL oxidized in the presence of 10 μM-$CuCl_2$ for 24 h; 4, LDL modified simultaneously with 4 mM-HNE and 20 mM-MDA; 5, same as 2; 6, LDL modified by 20 mM-MDA.

Table 1. Changes in the relative electrophoretic mobility (REM) of aldehyde-treated LDL

The values for the REM were calculated by dividing the migration rate of the modified lipoprotein by that of the control.

Treatment of LDL	REM
Hexanal (4 mM)	1.22
Hepta-2,4-dienal (4 mM)	1.43
4-Hydroxyhexenal (4 mM)	1.28
4-Hydroxyoctenal (4 mM)	1.78
HNE	
1 mM	1.31
2 mM	1.60
3 mM	1.87
4 mM, +20 mM-MDA	3.30
MDA (20 mM	2.52

Table 2. Effect of $CuCl_2$-mediated oxidation of LDL on electrophoretic mobility (EM) and the generation of LPO and TBARS

LDL	EM (mm)	LPO (nmol/mg of LDL)	TBARS (nmol/mg of LDL)
Control	11	0	0.4
Oxidized (24 h, 10 μM-$CuCl_2$)	42	91.1	6.8
Oxidized (6 h, 50 μM-$CuCl_2$)	31	121.3	11.3
Oxidized (24 h, 50 μM-$CuCl_2$)	46	0	7.7

4 mM-MDA (results not shown) or 20 mM-MDA did not react with the antiserum (Fig. 1*b*). In order to study the formation of new epitopes on the oxidized form of LDL the lipoprotein was incubated in the presence of $CuCl_2$. The changes in the relative electrophoretic mobility as well as the generation of LPO and TBARS were measured as described in Table 2. Fig. 1(*b*) depicts the double-diffusion analysis of LDL oxidized with 10 μM-$CuCl_2$ for 24 h. The latter is compared with LDL treated with HNE alone and LDL treated simultaneously with HNE and MDA. In the double-diffusion analysis, oxidized LDL, HNE-treated and HNE/MDA-treated LDL showed a reaction of complete identity. It should be mentioned here that the treatment of LDL with HNE above 3–4 mM causes a turbidity of the lipoprotein solution. In the presence of MDA this effect was abolished. Even at HNE concentrations up to 8 mM, LDL stayed in solution. Without MDA, however, almost all LDL was aggregated [8,9,17,18]. We showed previously that, apart from HNE and MDA, several other aldehydes are generated in LDL upon its oxidation [9]. Thus we used hexanal, hepta-2,4-dienal and the structurally related aldehydes 4-hydroxyhexenal and 4-hydroxyoctenal for modification of LDL in order to test if our antiserum showed any cross-reactivity with LDL modified by these aldehydes. The increase in the electrophoretic mobility of the samples is shown in Table 1. Only 4-hydroxyoctenal-treated LDL showed a very faint reaction (weaker than LDL modified with 1 mM-HNE) in the double-diffusion analysis. This result was not surprising, since LDL modified by 4-hydroxyoctenal showed a change in its electrophoretic mobility comparable with HNE-treated LDL. Furthermore, 4-hydroxyoctenal differs from HNE only by one CH_2 group. As shown previously, however, the amount of 4-hydroxyoctenal was only 28.5% of the amount of HNE generated in copper-oxidized LDL [19]. As to the results, in our opinion the positive reaction obtained in the double-diffusion analysis with our antiserum against oxidized LDL was mainly due to the formation of HNE-derived epitopes on the surface of LDL. Recently, two papers were published that dealt with immunochemical detection of proteins altered by products of lipid peroxidation in the aorta of Watanabe rabbits. Using a monoclonal antibody against MDA-modified LDL, Haberland *et al.* [20] detected MDA-modified protein in atheromas. However, this antibody failed to react with copper-oxidized LDL. In a study by Palinski *et al.* [21] atherosclerotic lesions of Watanabe rabbits and fragments of apoB of LDL, extracted from human aorta, proved to stain positively with polyclonal antibodies raised in the guinea pig against autologous LDL, modified either with MDA or HNE, recognizing MDA-lysine or HNE-lysine adducts. Their antibody against MDA-modified LDL recognized copper-oxidized LDL too [21]. Yet our findings emphasize that the new epitopes created during lipid peroxidation on the surface of LDL also derive from HNE and possibly, but to a minor extent, 4-hydroxyoctenal.

Another purpose of our study was to investigate whether Lp(a) and VLDL, the other two lipoproteins also exerting a certain atherogenic potential, are susceptible to oxidation and whether new epitopes recognized by our antibody are formed on the surface of these lipoproteins. Therefore Lp(a) and VLDL were subjected to $CuCl_2$-mediated oxidation under conditions similar to those used for the oxidation of LDL. The oxidation of these lipoproteins was performed for periods of both 6 and 24 h in the presence of 5 μM-, 10 μM- (results not shown) and 50 μM-$CuCl_2$. The progress of oxidation was monitored by determination of LPO and TBARS and observation of changes of the electrophoretic mobility (Tables 3 and 4). In our opinion these two lipoproteins may also undergo structural and functional changes, as has been described for LDL [22,23]. Analysis of oxidized Lp(a) and VLDL by double immunodiffusion showed a positive reaction with the antiserum against HNE-modified LDL (Figs. 2*a* and 2*b*). This would imply that lipoproteins extracted from human aorta and staining positively with an antiserum against HNE-treated LDL

Table 3. Effect of $CuCl_2$-mediated oxidation of Lp(a) on electrophoretic mobility (EM) and the generation of LPO and TBARS

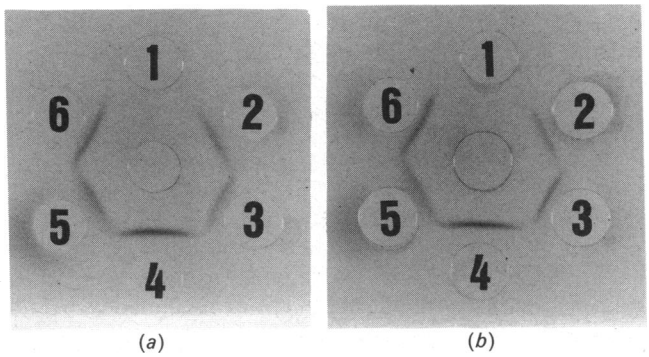
The concentration of Lp(a) was 1 mg/ml. The oxidation was performed in the presence of 50 μM-$CuCl_2$.

Lp(a)	EM (mm)	LPO [nmol/mg of Lp(a)]	TBARS [nmol/mg of Lp(a)]
Control	10.5	3.1	Not examined
Oxidized (6 h)	39.0	97.2	8.6
Oxidized (24 h)	> 50.0	0	5.8

Table 4. Effect of $CuCl_2$-mediated oxidation of VLDL on electrophoretic mobility (EM) and the generation of LPO and TBARS

The concentration of VLDL was 3 mg/ml. The oxidation was performed in the presence of 50 μM-$CuCl_2$.

VLDL	EM (mm)	LPO (nmol/mg of VLDL)	TBARS (nmol/mg of VLDL)
Control	13	5.1	0.3
Oxidized (6 h)	24	52.8	12.1
Oxidized (24 h)	43	22.1	9.9



(*a*) (*b*)

Fig. 2. Double-diffusion analysis of oxidized Lp(a), oxidized VLDL and modified and oxidized LDL with the antiserum raised against LDL modified by HNE

The antiserum was applied in the middle well. A 6 μg portion of lipoprotein protein was applied to each well (1–6). (*a*) Well 1, control Lp(a); 2, LDL modified by 3 mM-HNE; 3, Lp(a) oxidized in the presence of 50 μM-$CuCl_2$ for 6 h; 5, LDL modified simultaneously with 4 mM-HNE and 20 mM-MDA; 6, Lp(a) oxidized in the presence of 50 μM-$CuCl_2$ for 24 h. (*b*) Well 1, control VLDL; 2, LDL modified by 3 mM-HNE; 3, VLDL oxidized in the presence of 50 μM-$CuCl_2$ for 6 h; 4, LDL oxidized in the presence of 50 μM-$CuCl_2$ for 6 h; 5, LDL modified simultaneously with 4 mM-HNE and 20 mM-MDA; 6, VLDL oxidized in the presence of 50 μM-$CuCl_2$ for 24 h.

[21] could also at least in part stem from oxidized Lp(a) or VLDL. The question remains as to whether an oxidative modification of Lp(a) and VLDL might lead to an increase in the atherogenicity of these lipoproteins, as has been suggested for LDL in a recent review [24].

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