Oxidation of high-density lipoprotein HDL₃ leads to exposure of apo-AI and apo-AII epitopes and to formation of aldehyde protein adducts, and influences binding of oxidized low-density lipoprotein to type I and type III collagen *in vitro*¹

Joachim GREILBERGER and Günther JÜRGENS²

Institute of Medical Biochemistry, Karl-Franzens Universität Graz, Harrachgasse 21, A-8010 Graz, Austria

The changes in the immunological properties of apolipoprotein AI (apo-AI) and AII (apo-AII) during the oxidation of the high-density lipoprotein HDL₃ and its influence on the binding of heavily oxidized low-density lipoprotein (LDL) to type I and III collagen were investigated. Oxidation of HDL₃ or Eu³⁺-labelled HDL₃ was performed with CuSO₄, varying the time of oxidation. Oxidation of HDL₃ resulted in an increase in lipid hydroperoxides and enhanced the negative charge of this lipoprotein. Immunological studies with a solid-phase sandwich immunoassay revealed a strong increase in binding of Eu³⁺-labelled HDL₃ to polyclonal antibodies against apo-AI and apo-AII within the first 4 h of oxidation. Neo-epitopes were also formed by interaction of the apolipoproteins with degradation products from the lipid peroxidation of polyunsaturated fatty acids, as evidenced by an immunoreaction of oxidized Eu³⁺-labelled HDL₃ with antibodies to 4-hydroxynonenal (4-HNE)- and malondialdehyde (MDA)-

protein adducts. Western blot analysis of oxidized HDL₃ samples showed, as well as apo-AI and apo-AII bands, larger aggregated apolipoproteins, occurring after 0.5–2.5 h of oxidation. These aggregates were recognized by antibodies to apo-AI and apo-AII as well as by antibodies to 4-HNE- and MDA-protein adducts. Furthermore the original apo-AI monomers and apo-AII dimers decreased during the oxidation. The ability of native and oxidized HDL₃ to prevent the binding of Eu³⁺-labelled 24 h-oxidized LDL to collagen on microtitration plates was estimated. Interestingly, 2 h-oxidized HDL₃ competed most with the binding of 24 h-oxidized LDL on collagen type I and type III, followed by native HDL₃. However, 24 h-oxidized HDL₃ was a weaker competitor. Thus oxidative modification of HDL₃ strongly alters the immunological properties of this lipoprotein and its binding affinity for collagen.

INTRODUCTION

High-density lipoproteins (HDLs) are considered to have an important role in the reverse transport of cholesterol from the peripheral tissue to the liver [1–5]. This class of lipoproteins is probably able to counteract the accumulation of low-density lipoprotein (LDL) in the arterial intima, which is a key event in early atherogenesis [6]. LDL is assumed to become mildly oxidized by endothelial cells when entering the arterial wall and to recruit monocytes, which become resident macrophages [7]. These cells, as well as smooth-muscle cells, are able to generate free radical species, which in turn oxidatively modify LDL severely to a form recognized by scavenger receptors [8,9]. Accumulation of lipids in macrophages leads to an enhanced transformation of these cells to the so-called foam cells, which are typical cells in early atherosclerotic lesions [10]. In contrast, it was shown that HDL added to the medium of cultured lipid-laden foam cells [6] led to a significant efflux of cholesterol from these cells. However, an oxidative environment in the sub-endothelial space could also cause oxidative modifications of HDL because this lipoprotein was not found to be as well protected against copper-mediated oxidation as LDL. Oxidative modifications might have important consequences for the biological role of HDL in atherosclerosis. Nagano et al. [11] reported that after oxidative modification HDL lost the ability to stimulate the efflux of cholesterol from foam cells. Furthermore on oxidation the enzymes associated with HDL, such as paraoxonase

and platelet-activating factor acetylhydrolase might lose their abilities to protect LDL against oxidation [12] by metabolizing lipid hydroperoxides. Watson et al. [13] proposed that HDL removes oxidized phospholipids from oxidized LDL and platelet-activating factor acetylhydrolase on HDL hydrolyses them into lysophosphatidylcholine and fatty acid fragments. Furthermore HDL replenishes mildly oxidized LDL with platelet-activating factor acetylhydrolase, inactivating oxidized phospholipids and transferring them to HDL. An inhibition of lecithin:cholesterol acyltransferase was also obtained after modification of HDL apolipoproteins by aldehydes stemming from lipid peroxidation [14,15]. Although several articles deal with the oxidation of HDL, the information available about the changes in this lipoprotein on a molecular basis is much less than that on LDL. Thus the aim of the present study was to examine the change in the immunological properties of HDL on oxidation, especially the formation of aldehydic neo-epitopes on apolipoproteins AI and AII. In addition, we investigated how the oxidative modification of HDL would influence its ability to compete with the binding of oxidized LDL to collagen fibres.

MATERIALS AND METHODS

Materials

Anti-(rabbit IgG) and anti-(mouse IgG) were purchased from Sigma, rabbit antiserum against human apolipoprotein AI from

Abbreviations used: HDL, high-density lipoprotein; HNE, 4-hydroxynonenal; MDA, malondialdehyde; apo-AI and apo-AII, apolipoprotein AI and AII; EM, electrophoretic mobility.

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² To whom correspondence should be addressed.

Behring AG, sheep antiserum against human apolipoprotein AII from Immuno, and the Eu^{3+} -labelling kit (Delfia® no. 1244-302) from Wallac Oy. Type I and III collagen were prepared at the Department for Ophthalmology as described previously [16].

Lipoprotein preparation

LDL and HDL_3 were isolated from the plasma of normolipemic, fasting (12–14 h) young male donors with serum lipoprotein(a) levels lower than 1 mg/dl by differential ultracentrifugation with solid KBr to adjust the density. The following fractions were obtained. LDL and very-low-density lipoprotein were separated at a density of 1.063 g/ml (320 000 g, 24 h, 10 °C). LDL was separated from very-low-density lipoprotein by adjusting the density to 1.020 g/ml (320 000 g, 24 h, 10 °C). HDL_3 was separated from HDL_2 and lipoprotein-deficient serum at a density of 1.125 g/ml (320 000 g, 48 h, 10 °C). For further purification of HDL_3 one more centrifugation was performed at a density of 1.235 g/ml (320 000 g, 24 h, 10 °C). Kallikrein inactivator (aprotinin; 10^5 units/l; Bayer), Pefabloc (11.2 mg/l; Merck) and EDTA (1 g/l; Merck) were present during all steps of the LDL and HDL_3 preparation. HDL_3 was further purified on heparin-Sepharose CL-6B (Pharmacia Biotech) at 4 °C to remove the apolipoprotein E-containing fraction. Apolipoprotein E-free HDL_3 fractions were collected, concentrated by ultracentrifugation (100 000 g, 16 h, 10 °C) and stored at 4 °C. The purity of apolipoprotein E-free HDL_3 was checked by SDS/PAGE. The protein in LDL and HDL_3 was measured by the method of Lowry et al. [17]. The samples were sterile-filtered and stored at 4 °C in the dark until use.

Labelling of LDL and HDL_3 with Eu^{3+}

Eu^{3+} -labelling of LDL and HDL_3 was performed as described for LDL [16] in 50 mM NaHCO_3 , pH 8.3, containing 20 μM 2-carboxy-2,5,7,8-tetramethyl-6-chroman-ol (Trolox; Hofmann LaRoche). The lipoproteins (10 mg/ml total lipoprotein) were incubated with 0.3 mg of Eu^{3+} -(*p*-isothiocyanatobenzyl)-diethylenetriamine tetra-acetic acid (Delfia® Eu -labelling kit; Wallac Oy) at 25 °C in the dark for 12 h. Sephadex G-25 chromatography (Pharmacia Biotech) was used for the separation of the labelled lipoprotein fractions from free chelate in 50 mM Tris/HCl, pH 7.8, containing 0.05 % NaN_3 and 20 μM Trolox. The labelling yield of the Eu^{3+} -labelled lipoproteins was between 4 and 8 Eu^{3+} ions per protein molecule. The fluorescence intensity of Eu^{3+} -labelled lipoproteins was stable for more than 2 months. The labelled lipoproteins were used within 6 weeks.

Cu^{2+} -mediated oxidation of HDL_3 , Eu^{3+} -LDL and Eu^{3+} - HDL_3

Before oxidation, HDL_3 , Eu^{3+} -LDL and Eu^{3+} - HDL_3 were dialysed against 0.01 M PBS, pH 7.4, which was carefully degassed and then saturated with nitrogen. Cu^{2+} -mediated oxidation of HDL_3 , Eu^{3+} - HDL_3 (0.75 mg/ml) and Eu^{3+} -LDL (0.345 mg/ml) was performed at 37 °C with 10 μM CuSO_4 . At intervals between 0 and 24 h the reaction was terminated by adding a stop solution to achieve a final concentration of EDTA of 2.7 mM. The samples were saturated with nitrogen and stored at 4 °C in the dark.

The degree of modification of the oxidized lipoproteins was estimated as the relative electrophoretic mobility, i.e. relative to the non-oxidized and unlabelled native LDL or HDL_3 , on agarose gels (1 %, w/v) at pH 8.05 with the Lipidophor-system (Immuno AG). Lipid peroxides were estimated by a spectrophotometric assay with iodide colour reagent (Merck) at 365 nm, as developed in this laboratory [18].

To check for possible changes in the fluorescence intensity of Eu^{3+} -LDL and Eu^{3+} - HDL_3 occurring during oxidation, the lipoprotein samples were applied to polystyrene microtitration plates (Maxisorb; Nunc) in several dilutions with the enhancement solution. The fluorescence counts were measured with the Delfia® research fluorimeter (Wallac Oy). Oxidation of Eu^{3+} -LDL or Eu^{3+} - HDL_3 weakened the fluorescence only slightly.

Polyclonal antibodies against apo-AI or apo-AII and against HNE- or MDA-protein adducts

Purification of antisera against apo-AI (Behring AG), and against HNE- and MDA-protein adducts [19,20], all from rabbit, and against apo-AII (Immuno), from sheep, was performed on a DEAE-Sepharose fast flow column (Pharmacia Biotech). After dialysis of the antisera against 50 mM Tris/HCl (pH 8.2)/40 mM NaCl, they were applied to the column and eluted with 50 mM Tris/HCl (pH 8.2)/40 mM NaCl, with monitoring at 280 nm. IgG fractions were collected, dialysed against 50 mM Tris/HCl, pH 8.2, containing NaN_3 at a final concentration of 0.01 %, and stored at 4 °C. The purity of all antibodies was checked by SDS/PAGE.

Solid-phase fluorescence immunoassay

To investigate the formation of oxidation-specific epitopes or the change of native epitopes on apo-AI and apo-AII during the oxidation of HDL_3 , an assay was performed as follows: microtitration plates were incubated with a solution of the antibodies (200 μl per well; 5 $\mu\text{g}/\text{ml}$) mentioned above in coating buffer [1.58 g/l Na_2CO_3 /2.93 g/l NaHCO_3 (pH 9.6)] for 16 h at 4 °C. After two washes with washing buffer [10 mM PBS (pH 7.4)/0.9 g/l NaCl/0.05 % (v/v) Tween-20/0.02 % NaN_3], Eu^{3+} - HDL_3 samples (5 $\mu\text{g}/\text{ml}$; 200 μl per well) diluted in 10 mM PBS, pH 7.4, containing 1 g/l EDTA, were added and incubated for 90 min at 25 °C. After six washes, the fluorescence of bound Eu^{3+} was measured in the presence of enhancement solution (200 μl per well). The fluorescence was measured with a 1234 Delfia® research fluorimeter (Wallac Oy).

Competitive solid-phase sandwich fluorescence assay of 24 h-oxidized Eu^{3+} -LDL on collagen type I and III with HDL_3

For the competitive solid-phase fluorescence sandwich assay, polystyrene microtitration plates were coated with the proteins (10 $\mu\text{g}/\text{ml}$ of type I and III collagen; 200 μl per well) in 10 mM PBS, pH 7.4, at room temperature overnight. After three washes with washing buffer, 100 μl of 10 $\mu\text{g}/\text{ml}$ 24 h-oxidized Eu^{3+} -LDL was mixed with 100 μl of 0, 2 and 24 h-oxidized Eu^{3+} - HDL_3 (0, 0.1, 10, 100 and 1000 $\mu\text{g}/\text{ml}$ HDL_3) and incubated in 10 mM PBS, pH 7.4, for 90 min at room temperature on a shaker. After six washes with washing buffer, Eu^{3+} was released with enhancement solution (200 μl per well; Wallac Oy) and fluorescence was measured with a Delfia® research fluorimeter (Wallac Oy).

Statistical evaluation of the data obtained was performed by means of Student's *t* test.

Electrophoresis and Western blot analysis

SDS/PAGE [12 % (w/v) gel] was performed under non-reducing conditions. Aliquots (15–20 μg) of native or oxidatively modified HDL_3 protein dissolved in sample buffer [0.075 M Tris, pH 8.8, containing 20 % (w/v) glycerol and 0.01 % Orange G (Aldrich)] were applied per lane. Electrophoresis was performed in a Mini Protean II electrophoresis chamber (60 min at 50 mA and 150 V; Bio-Rad). Transfer to nitrocellulose membranes (0.1 μm pore

size; Hoefer Scientific Instruments) was done with an LKB NovaBlot electrophoretic transfer kit (Pharmacia-LKB) for 90 min at 50 mA and 17 V. The transfer solution contained 48 mM Tris, 39 mM glycine, 0.037% SDS and 20% (v/v) methanol. Non-specific binding sites were blocked with TBS [3% (v/v) skimmed milk in 20 mM Tris/90 mM NaCl/1 mM NaN_3 (pH 7.4)] for 3 h at 25 °C. The nitrocellulose membranes were incubated at 4 °C overnight with the antibodies against apo-AI, apo-AII, HNE-protein and MDA-protein adducts (1:1000 dilution in TBS). After three washes with TTBS [TBS containing 0.05% (v/v) Tween-20], on the one hand alkaline phosphatase-conjugated anti-(rabbit IgG) (Sigma), which bound to anti-apo-AI, or horseradish peroxidase-labelled anti-(goat IgG) (Sigma), which bound to anti-apo-AII, was added for incubation at 25 °C for 3 h. After a further washing, bound rabbit IgG was revealed with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 1 M 2-amino-2-methyl-1-propane, pH 10.3, and bound goat antibody was detected with 25 mg of 4-chloro-1-naphthol in 47.5 ml of TBS containing 2.5 ml of ethanol and 100 μl of H_2O_2 . On the other hand, horseradish-peroxidase-labelled anti-(rabbit IgG) (Dako), which bound to anti-HNE-protein and anti-MDA-protein was added for incubation at 25 °C for 3 h. After a wash, enhanced chemiluminescence (ECL) of the revealed antibody was detected on a hyperfilm-ECL (Amersham Life Science).

RESULTS

Alterations of HDL₃ and Eu³⁺-HDL₃ during oxidation

Native HDL₃ and Eu³⁺-labelled HDL₃ (Eu³⁺-HDL₃) were oxidized in the presence of 10 μM CuSO_4 at 37 °C. After termination of the oxidation at intervals by the addition of EDTA, the degree of modification of oxidized Eu³⁺-HDL₃ was estimated as its electrophoretic mobility (EM) relative to non-modified or unlabelled native LDL, or by the content of lipid hydroperoxides (Figure 1). Although the EM (relative to native LDL) of Eu³⁺-

Figure 1 Changes in EM and changes in the content of lipid hydroperoxides of HDL₃ during a Cu²⁺-mediated (10 μM CuSO_4) oxidation at different periods up to 24 h

The degree of modification of oxHDL₃ was recorded by measuring the EM relative to native LDL ($\square$) or the content of lipid hydroperoxides ($\blacklozenge$). A total of four independent oxidation experiments with four different preparations of HDL₃ from four different donors was performed. The results are given as percentages, setting either the content of lipid hydroperoxides of 4 h-oxidized Eu³⁺-HDL₃ (55.2, 52.2, 53.8, 54.3 nmol/mg HDL₃) or the relative EM of 24 h-oxidized Eu³⁺-HDL₃ (relative EM, 4.4, 4.5, 4.4, 4.4) as 100%. Values are means $\pm$ S.D.

Figure 2 Immune reactivity of anti-(apo-AI), anti-(apo-AII), anti-(HNE-protein adducts) and anti-(MDA-protein adducts) to oxidized Eu³⁺-HDL₃ in a solid-phase fluorescence immunoassay

Eu³⁺-HDL₃ was oxidized with 10 μM CuSO_4 for different periods up to 24 h. After microtitration plates had been coated with antibodies (1 μg per well), oxidized Eu³⁺-HDL₃ (2 μg per well) samples were applied. A total of four independent oxidation experiments with four different preparations of HDL₃ from four different donors was performed. The results of binding of oxidized Eu³⁺-HDL₃ to anti-(apo-AI) ($\blacklozenge$) and anti-(apo-AII) ($\blacktriangle$) (upper panel) or anti-(HNE-protein adducts) ($\diamond$) and anti-(MDA-protein adducts) ($\triangle$) (lower panel) are given as percentages, setting the immune reactivity of 24 h-oxidized Eu³⁺-HDL₃ to 100%. Values are means $\pm$ S.D.

HDL₃ modified oxidatively for 1 h was not altered, the content of lipid hydroperoxides increased immediately after initiation of the oxidation, reaching a maximum concentration after 3–4 h. The EM increased slightly after 2 h, followed by a stronger increase up to 4 h, and reached a maximum at 24 h of oxidation, whereas lipid hydroperoxides decreased strongly between 4 and 24 h.

A similar increase in the relative EM and in the content of lipid hydroperoxides during oxidation was obtained with unlabelled HDL₃ under the same conditions of oxidation as for Eu³⁺-HDL₃.

Increase in the immune reactivity of HDL₃ with antibodies against apo-AI and apo-AII and against HNE- and MDA-protein adducts during oxidative modification

A Cu²⁺-mediated oxidation of LDL leads to the degradation of apolipoprotein B. The native epitopes decreased, as verified by a diminished reactivity to antibodies against native apolipoprotein B [16]. To estimate the influence of oxidation of HDL₃ on the immune reactivity of apo-AI and apo-AII, microtitration plates were coated with polyclonal antibodies against apo-AI and apo-AII. The wells were then incubated with Eu³⁺-HDL₃ samples oxidatively modified to different degrees. The immune reactivity of Eu³⁺-HDL₃ with antibodies against apo-AI and apo-AII strongly increased (by approx. 80%) within the first 4 h of

Figure 3 Western blot analysis of native and Cu^{2+} -oxidized HDL_3

The lipoproteins, oxidized for different periods at 37 °C, were subjected to non-reducing SDS/PAGE [12% (w/v) running gel] and transferred to nitrocellulose membranes. Western blotting was performed with anti-(apo-AI) and anti-(apo-AII) (top panel), anti-(MDA-protein adducts) (middle panel) and anti-(HNE-protein adducts) (bottom panel) (dilutions 1:1000). Horseradish peroxidase-conjugated anti-(rabbit IgG) (dilution 1:1200) for anti-(apo-AI), anti-(MDA-protein adducts) and anti-(HNE-protein adducts) or alkaline phosphatase-conjugated anti-(sheep IgG) (dilution 1:1200) for anti-(apo-AII) were used as the second antibodies.

oxidation, as shown in Figure 2 (upper panel). This increase was expressed more with the antibody against apo-AI than with that against apo-AII; a slight but equal increase with both antibodies was found up to 24 h of oxidation of Eu^{3+} - HDL_3 .

Simultaneously with the estimation of the immune reactivity against the antibodies against apo-AI and apo-AII the generation

of MDA and HNE epitopes on Eu^{3+} - HDL_3 during oxidation was followed with the respective antibodies (Figure 2, lower panel). After 4 h of oxidation approx. 60 % of newly generated aldehydic epitopes were present on Eu^{3+} - HDL_3 . The formations of MDA and HNE epitopes during the time course of oxidation were very similar.

Table 1 Semi-quantitative evaluation of the distribution of MDA- or HNE-derived epitopes on apo-AI and apo-AII of Cu²⁺-oxidized HDL₃ obtained by Western blot analysis shown in Figure 3

Symbols: + + +, band showing strongest reaction; + +, band showing strong reaction; +, band showing reaction; —, no reaction.

Epitopes	Apolipoproteins and aggregates	Oxidation time (h) ...	Epitopes									
			0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	24
MDA	AI monomer		—	—	—	—	—	—	—	—	—	—
	AI polymer		—	—	—	—	—	+	+	++	+++	+++
	AII dimer		—	—	—	—	—	—	—	—	—	—
	AII tetramer		—	+	+	++	++	++	++	++	+	—
	AII hexamer		—	+	++	++	++	++	++	+	+	+
	AI/AII (1:2)		—	—	—	+	+	+	+	—	—	—
	AI/AII (1:4)		—	—	—	—	—	—	—	—	—	—
HNE	AI monomer		—	—	—	+	++	++	++	+++	++	+
	AI polymer		—	—	—	—	++	++	+++	+++	+++	+++
	AII dimer		—	—	—	—	—	—	—	—	—	—
	AII tetramer		—	—	—	+	+++	+++	+++	+++	++	+
	AII hexamer		—	—	—	+	+++	+++	+++	+++	++	+
	AI/AII (1:2)		—	—	—	+	+++	+++	+++	++	+	++
	AI/AII (1:4)		—	—	—	—	—	—	++	+++	+++	+++

Recognition of aggregated and non-aggregated apolipoproteins of oxidized HDL₃ by antibodies against apo-AI and apo-AII, and against HNE- or MDA-protein adducts in Western blot analysis

Oxidative modification of HDL₃ was shown to cause the aggregation of apo-AI and apo-AII [21]. This experiment was performed to follow the intermolecular and intramolecular cross-linking and aggregation respectively of apo-AI and apo-AII on the one hand, and to study the expression of the aldehydic epitopes on the other. All differently oxidized HDL₃ samples were subjected to electrophoresis on 12% (w/v) denaturing polyacrylamide gels followed by Western blot analysis with the four antibodies used above. As shown in Figure 3 (top panel), anti-(apo-AI) and anti-(apo-AII) recognized the monomers of apo-AI (28 kDa) and the dimers of apo-AII (17 kDa) of native HDL₃, 0.5–4 h-oxidized HDL₃ with decreasing intensity, but after 24 h of oxidation no monomers of apo-AI and no dimers of apo-AII were seen on the blots. Bands of aggregated apo-AI and apo-AII arose after only 0.5 h of oxidation of HDL₃ in different patterns and with an intensity dependent on the oxidation of HDL₃. Apo-AII formed tetramers (34 kDa) and hexamers (51 kDa) at the highest levels between 1 and 3 h of oxidation, whereas apo-AI aggregated after 2 h to form one band at the top of the gel, which was most intense with 24 h-oxidized HDL₃. Bands with a molecular mass of 45 kDa consisting of both apo-AI and apo-AII at a molar ratio of 1:2 were generated between 0.5 and 4 h of oxidation. Bands revealing a molecular mass of 62 kDa represented adducts of apo-AI and apo-AII at a molar ratio of 1:4. They were detected after 2 h of oxidation, expressed at highest levels between 2.5 and 3.5 h and present to a smaller extent at 4 and 24 h of oxidation.

Each band of aggregated or non-aggregated apo-AI and apo-AII was tested with the antibodies against MDA- or HNE-protein adducts as shown in Figures 3 (middle panel) and 3 (bottom panel). A semi-quantitative evaluation is given in Table 1. HNE-derived epitopes were present on all bands except the apo-AII dimers. The strongest staining with the antibody against HNE-protein adducts was found on the apo-AII tetramers and hexamers and the apo-AI/AII adducts (molar ratio 1:2) between 2 and 3.5 h of oxidation, whereas the apo-AI/AII adducts

(molar ratio 1:4) revealed a strong staining between 3 and 24 h of oxidation. Furthermore the staining of the polymer of apo-AI increased strongly from 2 to 24 h of oxidation of HDL₃.

MDA-derived epitopes were present only on the apo-AI polymers, apo-AII tetramers and to a smaller extent on the apo-AI/AII adducts (molar ratio 1:2). The expression of epitopes derived from MDA, a divalent aldehyde capable of cross-linking proteins, was strongest on the apo-AII tetramers between 1.5 and 3.5 h of oxidation, on the apo-AII hexamers between 1 and 3 h of oxidation, and on the polymers of apo-AI from 3.5 to 24 h of oxidation of HDL₃.

Competition of binding of 24 h-oxidized Eu³⁺-LDL on collagen type I and III with differently oxidized HDL₃

It has been shown that strongly oxidatively modified LDL bound *in vitro* to a greater extent to connective-tissue proteins, such as collagen type I, III and V, than did native LDL [16,22]. To study the ability of HDL₃ to prevent the binding of strongly oxidized LDL to collagen, 24 h-oxidized Eu³⁺-LDL (relative EM 3.6) was mixed with differently oxidized HDL₃ samples in several concentrations and added to microtitration plates coated with type I and type III collagen. After a wash, the fluorescence in the wells was counted.

Table 2 shows the competition of 24 h-oxidized Eu³⁺-LDL with native, 2 h-oxidized and 24 h-oxidized HDL₃ on type I collagen. At each of the three concentrations of competitor applied (10, 100 and 1000 µg), 2 h-oxidized HDL₃ competed best with oxidized Eu³⁺-LDL (10 µg/ml). However, on further oxidation the competing ability of HDL₃ decreased. Furthermore native HDL₃ was also a good competitor.

With type III collagen (Table 2) similar results were obtained to those for collagen type I. Again, 2 h-oxidized HDL₃ was the strongest competitor for the binding of 24 h-oxidized Eu³⁺-LDL.

The results shown were obtained in nine independent experiments with HDL₃ from four preparations from four donors. Although native HDL₃ decreased the binding of 24 h-oxidized Eu³⁺-LDL to the collagen fibres, the 2 h-oxidized HDL₃ was an even better competitor. However, further oxidation of HDL₃ weakened the competitive capability of HDL₃.

Table 2 Composition of native, 2 h-oxidized and 24 h-oxidized HDL₃ with the binding of 24 h-oxidized Eu³⁺-LDL to collagen type I or type III in a competitive fluorescence assay

Microtitration plates were coated with each collagen phenotype at 2 µg per well. Samples (1 µg per well) of 24 h-oxidized Eu³⁺-LDL (relative EM to native LDL: 3.6) was added in the presence of increasing amounts of native HDL₃, 2 h-oxidized and 24 h-oxidized HDL₃ in 10 mM PBS, pH 7.4. The results are expressed as percentages of B/B_0 , where B is the amount of 24 h-oxidized Eu³⁺-LDL bound to type I or type III collagen in the presence of the competitor, and B_0 in its absence. Means $\pm$ S.D. for nine independent experiments are given for four different preparations of HDL₃ from four different donors. * Statistically significant ($P < 0.05$) difference from the values for native HDL₃; ** statistically significant ($P < 0.0001$) difference from the values for native HDL₃; n.s., no statistically significant difference from the values for native HDL₃; † statistically significant ($P < 0.0001$) difference from values for 2 h-oxidized HDL₃.

Collagen type	HDL ₃ (µg)	HDL ₃ type		
		Native HDL ₃	2 h oxidized	24 h oxidized
I	10	99.6 $\pm$ 2.0	78.6 $\pm$ 5.8**	99.4 $\pm$ 1.3n.s./†
	100	60 $\pm$ 2.9	56.6 $\pm$ 2.1*	91.7 $\pm$ 2.4**/†
	1000	45.2 $\pm$ 3.8	36.7 $\pm$ 1**	56.7 $\pm$ 2.8**/†
III	10	95.2 $\pm$ 2.4	86 $\pm$ 1**	94.8 $\pm$ 1.7n.s./†
	100	69.9 $\pm$ 3.5	53.9 $\pm$ 1.7**	78.1 $\pm$ 3.2**/†
	1000	46.1 $\pm$ 2.5	38.1 $\pm$ 1.7**	54.9 $\pm$ 3.9**/†

DISCUSSION

In the genesis of atherosclerosis a crucial role is attributed to LDL, HDL and the LDL/HDL ratio as these lipoproteins are responsible for the transport of cholesterol [23–25]. The present study has described for the first time the generation of aldehydic products on apolipoproteins of HDL₃ during Cu²⁺-mediated oxidation and investigated the ability of HDL₃ and oxidized HDL₃ to prevent the binding of strongly oxidatively modified LDL to collagen phenotypes of the arterial wall.

It is known that susceptibility of HDL to oxidation is higher than that of LDL, owing to the lack of antioxidants in nascent HDL, such as vitamin E, and the carriage of detectable amounts of lipid hydroperoxides [26]. Lipid hydroperoxides increased strongly during the first 2 h of oxidation with Cu²⁺, whereas only a small change in the relative EM of HDL₃ was obtained.

The immune reactivity of HDL₃ with the antibodies against apo-AI and apo-AII strongly increased within the first 4 h of oxidation. This enhanced immune reactivity might be due to the presentation of epitopes on the surface of HDL₃ that might have been buried in the lipid domain of this lipoprotein before oxidation. An increased immune reactivity of apo-AI with storage of human serum has been described [27]; atmospheric oxidation was assumed to be the cause. The oxidation of HDL with Fe²⁺/Fe³⁺ or Mn²⁺ was found to increase the immune reactivity of apo-AI 12–80-fold, whereas other divalent cations such as Cu²⁺ at 1 mM had minimal effects [28]. These results are in contrast with our results, yet it has to be taken into consideration that we used a concentration of 10 µM Cu²⁺ to achieve the effects described. Furthermore we also found an increase in the immune reactivity with anti-(apo-AII), which preceded the increase with anti-(apo-AI).

In addition to antibodies against apo-AI and apo-AII, we used antibodies against HNE- and MDA-modified apolipoproteins for the investigation of oxidatively modified HDL₃. HNE and MDA are breakdown products of lipid hydroperoxides and are highly reactive, especially with positively charged amino acids such as lysine, forming aldehydic epitopes [29]. The antibodies used in this study were made against HNE- or MDA-modified LDL [20]. However, they also reacted with HDL and other

proteins such as albumin after their modification with the respective aldehydes [19]. These results suggest that the antibodies are recognizing epitopes in general formed as HNE- or MDA-protein adducts. Both antibodies showed a strong increase in the immune reactivity with oxidatively modified HDL₃ within the first 4 h of oxidation, which was accompanied by an increase in EM relative to native HDL₃. It should be mentioned that Cu²⁺-oxidation or modification of HDL by aldehydes such as HNE, MDA or others were found to lead to an inhibition of the activation of lecithin:cholesterol acyltransferase [14,15], but the formation of HNE- or MDA-derived epitopes on oxidation of HDL₃, as obtained in the present study on apo-AI and apo-AII and their aggregates, was not shown. After only 2 h of oxidation of HDL₃ with Cu²⁺, more than 30 % of the totally formed new epitopes were present. To characterize the distribution of the aldehydic epitopes on the apolipoproteins, Western blot analysis was performed. HNE-derived epitopes were present on all apo-AI or apo-AII monomers or polymers except apo-AII dimers, whereas MDA-derived epitopes could be detected only on apo-AII tetramers and hexamers, apo-AI polymers and very weakly on mixed apo-AI/AII (1:2) aggregates. The reason that MDA-derived epitopes were present on neither apo-AI monomers nor apo-AII dimers might be that MDA, as a bivalent aldehyde, leads to cross-linking of the apolipoproteins, whereas HNE is a monovalent aldehyde.

Studies on the binding of HDL or oxidized HDL to extracellular matrix were of special importance because in an investigation by Vollmer et al. [30] a few deposits of apo-AI and apo-AII in the intima were observed with the first signs of atherosclerosis. Apo-AI and apo-AII were localized extracellularly, associated with the connective tissue. With progression of atherosclerosis the distributions of apo-AI, apo-AII and of apolipoprotein B changed. Apo-AI and apo-AII were located within the intimal layer intracellularly, mainly in foam cells, or extracellularly, whereas apolipoprotein B was found to be exclusively deposited extracellularly. Thus it was of interest to investigate *in vitro* whether HDL₃ or oxidized HDL₃ was able to bind to matrix proteins, especially to different collagen phenotypes such as LDL and oxLDL [16]. It has been assumed [31] that binding of negatively charged macromolecules such as LDL to the extracellular matrix could be followed by oxidation of the lipoprotein. Afterwards it would be taken up by macrophages via the scavenger receptor's gliding along the collagen fibrils. Although an increased binding of oxidized LDL to collagen type I, III and V, which tended to increase with the progression of atherosclerosis, has been reported [16,22], little is known about native and oxidatively modified HDL preventing the binding of oxidized LDL to those collagen phenotypes. At the moment we do not have an explanation why 2 h-oxidized HDL₃ competed with oxidized LDL for collagen type I and III better than native or 24 h-oxidized HDL₃ did, nor why this effect was stronger with collagen type III than with type I. The observations underline the assumption that certain newly formed epitopes on moderately oxidized HDL might be responsible for competition rather than a sole increase in negative charge of oxidized HDL.

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