Reagent or myeloperoxidase-generated hypochlorite affects discrete regions in lipid-free and lipid-associated human apolipoprotein A-I

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We have previously shown that the modification of high-density lipoprotein subclass 3 (HDL_3) by HOCl transformed an anti-atherogenic lipoprotein into a high-uptake form for macrophages and caused a significant impairment of cholesterol efflux capacity [Panzenboeck, Raitmayer, Reicher, Lindner, Glatter, Malle and Sattler (1997) J. Biol. Chem. **272**, 29711–29720]. To elucidate the consequences of treatment with OCl^- on distinct regions in apolipoprotein A-I (apo A-I), lipid-free and lipid-associated apo A-I were modified with increasing molar ratios of NaOCl or HOCl generated by the myeloperoxidase/H_2O_2/Cl^- system. CD analysis revealed a pronounced decrease in α-helicity for lipid-free apo A-I modified by NaOCl, whereas lipid-associated apo A-I was less affected. The modification of apo A-I by NaOCl (molar oxidant-to-lipoprotein ratio 6:1) resulted in the formation of two distinct oxidized forms of apo A-I with molecular masses 32 or 48 atomic mass units (a.m.u.) higher than that of native apo A-I, indicating the addition of two or three oxygen atoms to the native protein. HPLC analysis of tryptic digests obtained from lipid-free and lipid-associated apo A-I modified with increasing oxidant-to-apolipoprotein molar ratios revealed a concentration-dependent modification of apo A-I: at a low molar oxidant-to-lipoprotein ratio (5:1) the peaks corresponding to the methionine-containing tryptic peptides T11 (residues 84–88), T16 (residues 108–116) and T22 (residues 141–149), located in the central region of apo A-I, disappeared. Their loss was accompanied by the formation of three oxidation products with a molecular mass 16 a.m.u. higher than that of the native peptides. This indicates the addition of oxygen, most probably caused by the oxidation of Met^{86}, Met^{112} and Met^{148} to the corresponding methionine sulphoxides. At a molar NaOCl-to-apo A-I ratio of 10:1 the disappearance of peptides T1 (residues 1–10), T7 (residues 46–59) and T9 (residues 62–77) was accompanied by the occurrence of new peaks 33.5 and 33.1 a.m.u. higher than those of the native peptides. Amino acid analyses of peptides T7 and T9 after modification with NaOCl confirmed that Phe^{57} and Phe^{71} were primary targets for oxidation by HOCl. GLC–MS analysis of hydrolysates obtained from OCl^--modified T7, T9, apo A-I and HDL_3 confirmed that Phe residues are an early target for OCl^- modification. At molar NaOCl-to-apo A-I ratios of 25:1, the peak areas of peptides T31 (residues 189–195) and T32 (residues 196–206) decreased markedly. Most importantly, incubation of apo A-I with the myeloperoxidase/H_2O_2/Cl^- system (the source of HOCl *in vivo*) resulted in almost identical modification patterns to those observed with reagent NaOCl.

Key words: amphipathic helix, hypochlorous acid, primary structure, secondary structure.

INTRODUCTION

Apolipoprotein A-I (apo A-I) is a polypeptide of 243 amino acid residues and the major apolipoprotein of high-density lipoproteins (HDLs). High plasma levels of HDL are inversely correlated to the risk of developing coronary heart disease, an effect commonly attributed to the role of HDL in the removal of excess cholesterol by reverse cholesterol transport [1]. During this process, apo A-I is thought to be important owing to its ability to promote cholesterol efflux from peripheral cells and to activate lecithin:cholesterol acyltransferase (LCAT), the enzyme responsible for the esterification of newly acquired cholesterol [2]. Apo A-I contains tandem amino acid domains, which are arranged into amphipathic helices [3]. In particular the amphipathic nature of these helices is thought to contribute to the ability of apo A-I to associate with lipids and membrane surfaces, as well as to activate LCAT. Several distinct apo A-I epitopes responsible for the unique features were identified: defined amino acid stretches contribute to cellular cholesterol efflux [4,5] or LCAT activation [6]. The C-terminal part of apo A-I was shown to be responsible for association with lipids [7], whereas the N-terminal part seems to be responsible for stabilizing its lipid-free structure [8,9].

Many properties of apo A-I directly linked to the structural integrity of this apoprotein can be lost during oxidation and/or modification. Among the amino acids present in apo A-I, the three methionine residues of apo A-I (Met^{86}, Met^{112} and Met^{148}) located at non-polar faces of amphipathic helices seem to be particularly susceptible to oxidation. Two of them, Met^{112} and Met^{148}, are oxidized to the corresponding sulphoxides after treatment with H_2O_2 or chloramine [10,11]. As confirmed by CD measurements, the oxidation of Met^{112} and Met^{148} leads to a loss of α-helicity in apo A-I, indicating that alterations in the secondary structure are due to the formation of methionine sulphoxides [Met(SO)] [10]. Limited-proteolysis experiments have shown that the oxidation of Met^{112} and Met^{148} switches the susceptibility of apo A-I to protease from the N-terminus to the C-terminus [7]. These findings underline that methionine

Abbreviations used: a.m.u., atomic mass unit; apo, apolipoprotein; DMS, dimethyl suberimidate; DPPC, dipalmitoylphosphatidylcholine; FCS, fetal calf serum; HDL_3, high-density lipoprotein subclass 3; LCAT, lecithin:cholesterol acyltransferase; LC–ESI–MS, liquid chromatography–electrospray ionization MS; mAb, monoclonal antibody; MPO, myeloperoxidase; NICI, negative-ion chemical ionization; rHDL, reconstituted high-density lipoprotein; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; TIC, total ion current.

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oxidation is important for defining the conformation in lipid-free and lipid-associated apo A-I. Recently the formation of two specific oxidized forms of apo A-I, containing one or two Met(SO) groups during the early stages of Cu^{2+} or soybean lipoxygenase oxidation was reported [12,13].

Among numerous oxidants generating modified and/or oxidized lipoproteins *in vitro*, HOCl is of potential relevance *in vivo*. HOCl is formed by the myeloperoxidase (MPO)/H_2O_2/Cl^- system from activated neutrophils and/or monocytes [14]. The importance of MPO as a potential oxidant *in vivo* is underlined by the presence of enzymically active MPO in human atherosclerotic lesions [15] and the presence of HOCl-modified (lipo)proteins in advanced human atherosclerotic lesions [16]. The MPO/H_2O_2/Cl^- system converts tyrosine residues to tyrosyl radicals, which undergo dimerization reactions to forming stable dityrosine, trityrosine and pulcherosine [17]. *o*,*o*′-Dityrosine was also suggested as a specific marker for tyrosyl-radical-mediated lipoprotein modification in atherosclerotic lesions [18]. Another potential marker for HOCl-derived protein oxidation is 3-chlorotyrosine [19]. In addition to its chlorinating capacity the MPO/H_2O_2/halide system oxidizes α-amino acids to a family of aldehydes via the decomposition of unstable α-chloramines [20]. Aldehydes generated by that route could then covalently modify amino acid residues on proteins, lipids and nucleic acids [20].

During the present study we intended to investigate the effect(s) of reagent NaOCl and HOCl, the latter generated via the MPO/H_2O_2/Cl^- system, on structural features of lipid-free and lipid-associated apo A-I with the use of CD, immunoblotting, HPLC and electrospray ionization (ESI)–MS techniques. To investigate further whether distinct regions of this apoprotein are affected by oxidation with OCl^- and to characterize some oxidation products, we prepared tryptic digests of native and OCl^--oxidized apo A-I. The resulting peptide fragments were subsequently characterized by liquid chromatography–ESI–MS (LC–ESI–MS) and amino acid analysis. Hydrolysates obtained from oxidized peptides, apo A-I and HDL subclass 3 (HDL_3) were further characterized by GLC–MS.

EXPERIMENTAL

Materials

NaOCl, dipalmitoyl phosphatidylcholine (DPPC), sodium cholate, 4-chlorophenylalanine, heptafluorobutyric acid anhydride, organic solvents and KBr were purchased from Sigma. Dimethyl suberimidate (DMS) was obtained from Pierce. MPO (isolated from human leucocytes) was purchased from Alexis Biochemicals. Trypsin (sequencing grade) was obtained from Boehringer Mannheim. C_{18} Sep-Pak solid-phase extraction columns (1 ml bed volume) were from Waters.

Isolation of human apo A-I

HDL was isolated from plasma obtained from normolipaemic volunteers by sequential ultracentrifugation, then delipidated as described [21]. The delipidated apoproteins were redissolved in 50 mM glycine/4 mM NaOH/0.5 M NaCl/6 M urea (pH 8.8). HDL apoproteins were then separated by size-exclusion chromatography on a Sephacryl S-200 column (3 cm × 150 cm) as described [21]. The fractions containing the major peak were pooled, dialysed against NH_4HCO_3 and freeze-dried. The product was homogeneous as assessed by SDS/PAGE, reverse-phase HPLC (RP-HPLC) (see below) and amino acid analysis (see below).

Modification of apo A-I with NaOCl

To prepare oxidized apo A-I, 1 mg of protein/ml of PBS (10 mM, pH 6) was incubated with NaOCl [22] at the indicated molar ratio of NaOCl to apo A-I. The concentration of NaOCl was calculated by using a molar absorption coefficient for NaOCl of 350 $mol^{-1} \cdot cm^{-1}$ at 292 nm. NaOCl was added once and the mixture was gently vortex-mixed, then kept on ice for 30 and 90 min at 37 °C. Modified apo A-I was dialysed overnight against water at 4 °C before use. During all experiments in which the modified products were analysed by HPLC or LC-MS, the ratio of molar oxidant to apo A-I was kept between 5:1 and 25:1 because higher modification rates severely hampered subsequent HPLC analyses.

Modification of apo A-I by MPO

To 1 mg of apo A-I/ml of PBS (50 mM, pH 7.4), 15 additions of 20 μM H_2O_2 were made at 10 min intervals at 37 °C. MPO (25 units/ml, 13 nM) was added at the start and subsequently at every second addition of H_2O_2 [22]. At alternate additions of H_2O_2, 2 μM ascorbate was added to recycle inactivated MPO. The reaction mixture was incubated for 1 h at 37 °C and dialysed overnight against water at 4 °C before use. MPO activity was measured as described [23].

SDS/PAGE and Western blotting

Apo A-I samples were run for 90 min at 150 V in a miniprotean chamber (Bio-Rad, Vienna, Austria). Proteins were transferred electrophoretically to nitrocellulose membranes (150 mA, 4 °C, 90 min). NaOCl-modified apo A-I was detected with the monoclonal antibody (mAb) clone 2D10G9 [24], dilution 1:50, followed by a horseradish-peroxidase-conjugated anti-mouse IgG (AP181 P; Biomedica), dilution 1:5000. For the detection of native apo A-I, a polyclonal rabbit anti-(human apo A-I) antibody was used [22], followed by a horseradish-peroxidase-conjugated goat anti-rabbit IgG (Sigma). Detection was performed by enhanced chemiluminescence.

Preparation of reconstituted HDL (rHDL)

rHDL was prepared by the sodium cholate dialysis method with DPPC, apo A-I and sodium cholate in molar ratios of 120:1:100 as described [25]. The buffer used during the whole preparation was 10 mM Tris/HCl, pH 8.0, containing 0.15 M NaCl, 0.005 % EDTA and 1 mM NaN_3. In brief, 14.2 mg of lipid was dissolved in 1 ml of buffer containing 6.2 mg sodium cholate. The mixture was stirred at 40 °C for 90 min. After the addition of apo A-I (4 mg/1.4 ml) and stirring at 40 °C (20 min) the mixture was incubated at 37 °C for 16 h. Dialysis with exhaustive changes of buffer was performed at ambient temperature for 3 days. Samples were kept under N_2 and protected from light. rHDL was reisolated by density-gradient ultracentrifugation with a TLA 100.4 rotor (Beckman) [26].

Characterization of rHDL

The chemical composition was obtained by protein determination with the Lowry procedure [27]; the phospholipid content was measured enzymically (BioMerieux, Hazelwood, MO, U.S.A.). The content of apo A-I molecules/rHDL was obtained by cross-linking with DMS as described by Swaney [28]: 20 mg of DMS

was dissolved in 1 ml of 1 M triethanolamine, pH 9.7. The solution was added to the sample at a ratio of 10:1 (v/v) and a protein concentration of 1 mg/ml. The reaction was performed at ambient temperature for 90 min. The cross-linked products were separated by SDS/PAGE [5–20 % (w/v) gel] and revealed with Coomassie Blue.

Proteolysis experiments

Apo A-I and the rHDL preparations were dissolved in 0.1 M NH_4HCO_3 (containing 2.5 M urea) at a protein concentration of 0.6 mg/ml. The pH was kept between 7.5 and 8.5. Trypsin was added at a trypsin-to-apo A-I ratio of 1:25 (w/w) and the mixture was incubated at 37 °C for 24 h. The peptide fragments obtained from 100 μg of digested protein were separated by RP-HPLC on a Vydac C_{18} column (4.6 mm × 150 mm) with a gradient of water/0.1 % (v/v) trifluoroacetic acid (TFA) and acetonitrile/0.1 % (v/v) TFA [0–60 % (v/v) acetonitrile in 60 min], with detection by UV absorption at 215 nm. Identification by LC–ESI–MS was performed as described below.

LC–ESI–MS

Online MS analyses of proteolytic digests were performed on an LC–MS combination consisting of a Flux Rheos 4000 binary gradient pump (Munich, Germany), an ERC vacuum degasser (Spectronex, Vienna, Austria), a Vydac C_{18} column (4.6 mm × 150 mm) and a Finnigan LCQ ion-trap mass spectrometer equipped with an electrospray source (San Jose, CA, U.S.A.) under the chromatographic conditions described above. The settings of the ion source were: spray voltage 3.5 kV, capillary temperature 220 °C, capillary voltage 13.6 V and tube lens offset 30 V. Nitrogen was used as sheath gas at a flow rate of 100 units. The mass range was set to 400–2000 and each scan was averaged from three microscans. Peak monitoring was performed at $[MH^+]$ and $[MH^{2+}/2]$ atomic mass units (a.m.u.) because the ion current at $[MH^{2+}/2]$ was higher than at $[MH^+]$.

HPLC separation and ESI-MS of intact apo A-I

Native or modified apo A-I (5 μg) was separated by RP-HPLC on a Vydac C_{18} column by using the gradient described in [10]. The resulting peaks were collected manually during multiple HPLC runs and pooled. Samples were injected in the moving solvent [10 μl/min; water/acetonitrile (1:1, v/v)/0.05 % (v/v) TFA]. The ion trap settings were identical with those described above.

CD measurements

The CD spectra were recorded on a JASCO J715 spectropolarimeter that had been wavelength calibrated with neodymium glass in accordance with the manufacturer's suggestions. Standardization was performed with an aqueous solution of 0.06 % ammonium *d*-(+)-10-camphorsulphonate supplied by JASCO at a wavelength of 291 nm and with a path length of 0.1 cm. Apo A-I and rHDL samples (1 mg of protein/ml of PBS) were measured at ambient temperature in continuous-scan mode with a 1 nm bandwidth (50 nm/min) and a response of 1 s (response wavelength width 0.83 nm). A cell with a path length of 0.1 cm was used and spectra were signal-averaged by accumulating at least three scans. The spectra were normalized to molar residue ellipticity with the use of a mean residue molecular mass of 115.2 Da for apo A-I [8].

Amino acid analysis

Tryptic apo A-I fragments were collected manually from the RP column, dried under vacuum and redissolved in water. A 20 μl portion of the solution (containing approx. 0.5 μg of the peptide) was dried in a microhydrolysis apparatus under vacuum and hydrolysed with 200 μl of 6 M HCl at 116 °C for 24 h. After hydrolysis, samples were dried under vacuum. Derivatization of hydrolysed samples was performed with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as derivatizing agent in aqueous borate buffer, as described by the manufacturer (Waters). This derivatization procedure provides sensitivity in the low picomolar range for amino acid analysis. The fluorescent derivatives were analysed on a Waters Alliance system equipped with an AccQTag amino acid column (C_{18} material, 3.9 mm × 150 mm; Waters) and a fluorescence detector (λ_{ex} 250 nm, λ_{em} 395 nm). Tryptophan fluorescence was measured at 335 nm by using an excitation wavelength of 280 nm [29].

GLC–MS

For GLC–MS analysis, peptides T7, T9 and apo A-I were modified with reagent NaOCl as described above, whereas HDL_3 was modified with the $MPO/H_2O_2/Cl^-$ system. Peptides (T7 and T9), apo A-I and HDL_3 were hydrolysed in 6 M HBr/1 % (w/v) phenol at 120 °C for 24 h to avoid the formation of chlorinated amino acids during hydrolysis [30]. The hydrolysate was then dried, passed over a C_{18} solid-phase extraction column and the eluted amino acids were converted to the corresponding n-propyl esters (3.5 M HBr in propan-1-ol, 65 °C, 1 h). The reaction products were dried and the corresponding *N*-heptafluorobutyryl derivatives were generated by the addition of 50 μl of ethyl acetate/heptafluorobutyric acid anhydride (3:1, v/v) at 65 °C (10 min), as described [30]. Samples were analysed on a Trace gas chromatograph coupled to a Finnigan Voyager quadrupole mass spectrometer (ThermoQuest) and a Trace GLC machine with a Finnigan GCQ Ion Trap mass spectrometer (ThermoQuest). The GLC machines were equipped with a DB-5MS fused silica capillary column (15 m × 0.25 mm internal diam., 0.25 μm film thickness) from ThermoQuest. The injector was operated in the splitless mode at 280 °C. Helium was used as carrier gas. Initial column temperature was 80 °C for 1 min, followed by an increase of 30 °C/min to 310 °C followed by an isothermal hold until elution was complete. The mass spectrometer transfer line was kept at 310 °C. Negative-ion chemical ionization (NICI) was performed with methane as moderating gas at an electron energy of 70 eV and an emission current of 0.135 A. Electron impact (EI) mass spectra were recorded at an electron energy of 70 eV. Selected reaction monitoring was accomplished by ion-trap tandem MS, using the EI fragment ion at m/z 350 as a precursor ion and scanning the product ions.

RESULTS

rHDL preparations

Lipid-associated apo A-I was prepared by the sodium cholate dialysis method in the presence of DPPC as described [25]. The preparations re-isolated by ultracentrifugation contained three major rHDLs with Stokes radii of 18, 15 and 11 nm as analysed by non-denaturing gradient gel electrophoresis with the 11 nm

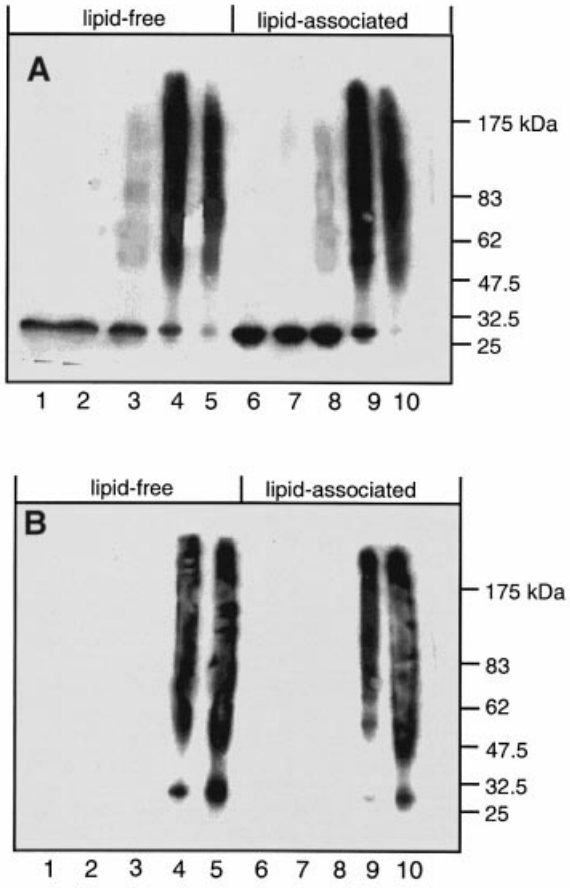

Figure 1 Detection of native and NaOCl-modified lipid-free and lipid-associated apo A-I by Western blots

Native and NaOCl-modified, lipid-free and lipid-associated apo A-I (1 μg) were separated on 5–25% gradient gels under denaturating conditions. Proteins were transferred to nitrocellulose as described in the Experimental section. Apo A-I was detected with rabbit anti-(human apo A-I) (**A**) and OCl^--modified epitopes present on apo A-I were detected with mAb 2D10G9 [24] (**B**) and detected as described in the Experimental section. Apo A-I was modified at molar oxidant ratios of 0, 5:1, 10:1, 25:1 and 50:1 either lipid-free (lanes 1–5) or associated with DPPC (lanes 6–10).

population contributing approx. 70%. The stochiometry of apo A-I in the re-isolated particles was determined by chemical cross-linking with DMS [28]. In line with other reports we have observed the presence of 2, 3 and 4 apo A-I molecules per rHDL particle [7]. These different populations were not separated for further modification experiments.

SDS/PAGE and immunoblot analysis

To identify effects of modification with NaOCl on the molecular mass distribution of lipid-free and lipid-associated apo A-I, the protein was modified with increasing NaOCl concentrations and separated by SDS/PAGE [5–25% (w/v) gradient gels]. Detection of apo A-I and OCl^--modified protein was performed with a polyclonal anti-(apo A-I) and a mAb (clone 2D10G9) that specifically recognized OCl^--modified epitopes on (apo)proteins [24]. Modification with OCl^- before immunoblotting revealed the formation of high-molecular-mass apo A-I aggregates at NaOCl-to-apo A-I molar ratios of at least 25:1 in lipid-free (Figure 1A, lanes 4 and 5) and lipid-associated (Figure 1A, lanes

Table 1 α-Helical content of native and NaOCl-modified lipid-free and lipid-associated apo A-I

Lipid-free and lipid-associated apo A-I were modified with the indicated molar ratios of NaOCl to apo A-I. The α-helical content was obtained by CD measurements as described in the Experimental section and calculated with the algorithm proposed by Yang et al. [31]. A mean molar residue molecular mass of 115.2 Da for apo A-I was used [8]. Results are means for two experiments.

Molar ratio of NaOCl to apo A-I	α-Helicity (%)	
	Lipid-free	Lipid-associated
0	33.8	78.3
5	28.8	79.0
10	23.2	77.9
25	20.6	75.6
50	18.8	67.0

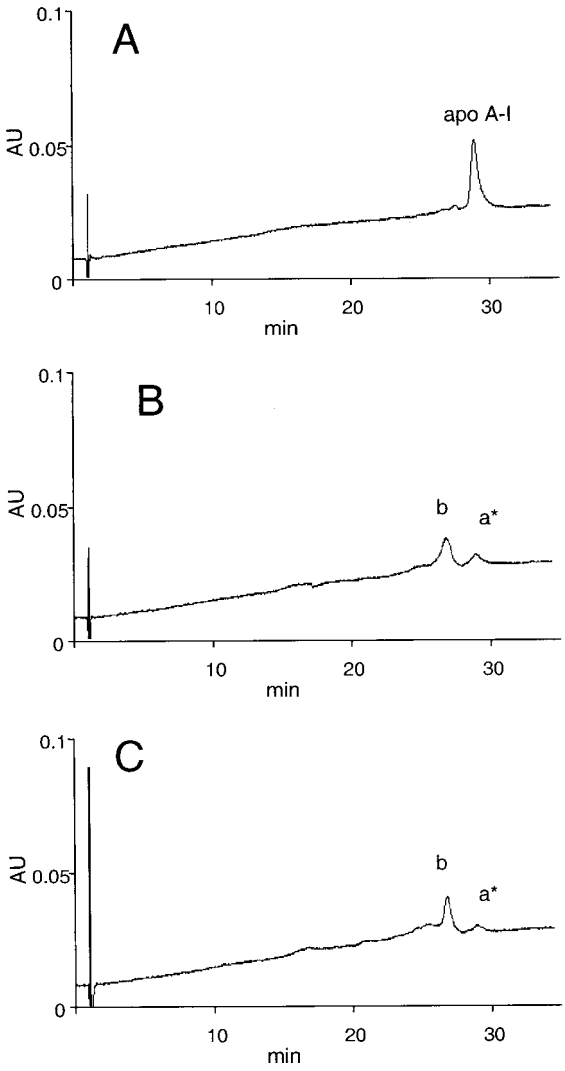

Figure 2 HPLC chromatograms of native, NaOCl- and MPO-oxidized apo A-I

Apo A-I was modified with a 6-fold molar excess of reagent NaOCl and MPO-derived HOCl as described in the Experimental section. Samples (5 μg) were separated by RP-HPLC as described in the Experimental section. Native apo A-I eluted as a single peak (**A**); after modification with reagent NaOCl (**B**) and MPO-derived OCl^- (**C**) two new products labelled with a* and b occurred.

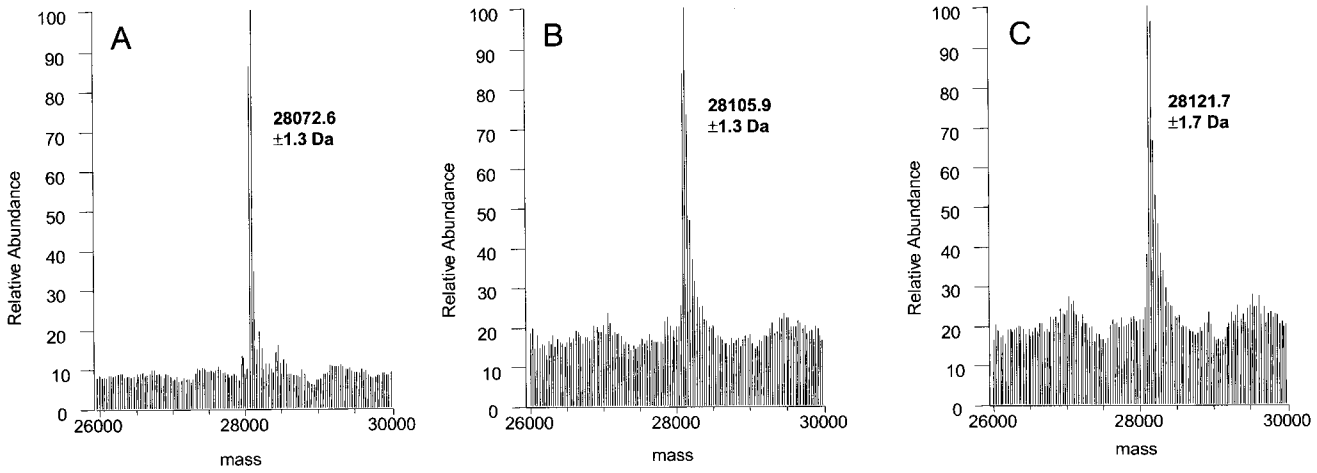

Figure 3 ESI-MS spectra of native and NaOCl-modified apo A-I

Native and NaOCl-modified apo A-I were separated under the conditions described in the legend to Figure 2. The resulting peaks were collected manually during multiple HPLC runs and analysed by ESI–MS (as described in the Experimental section). Representative deconvoluted ESI–MS spectra of native apo A-I (**A**), peak a* (**B**) and peak b (**C**) are shown.

9 and 10) apo A-I. Incubation of the blots with clone 2D10G9 revealed that this mAb did not cross-react with native apo A-I (Figure 1B, lanes 1 and 6) or with apo A-I modified with a 5- or 10-fold molar excess of NaOCl (Figure 1B, lanes 2, 3, 7 and 8). However, at NaOCl-to-apo A-I molar ratios of at least 25:1, 2D10G9 reacts with monomeric and aggregated NaOCl-modified lipid-free or lipid-associated apo A-I (Figure 1B, lanes 4, 5, 9 and 10).

CD studies

To evaluate the effects of modification with OCl^- on the secondary structure of lipid-free and lipid-associated apo A-I, far-UV CD spectra were obtained. The α-helical content was calculated as described [31]. Apo A-I modified with increasing NaOCl concentrations revealed a concentration-dependent loss of α-helicity. The α-helical content of lipid-free and lipid-associated apo A-I is summarized in Table 1. The additional α-helicity of lipid-associated compared with lipid-free apo A-I is in line with previous studies [7,8,32–34]. In general, lipid-free apo A-I was more sensitive to NaOCl treatment in terms of α-helicity than was lipid-associated apo A-I, a fact reflected by a pronounced loss of α-helicity, even at low molar NaOCl-to-apo A-I ratios. Whereas the α-helical content of lipid-free apo A-I was significantly decreased at an oxidant-to-protein molar ratio of 5:1, alterations in α-helicity of lipid-associated apo A-I became obvious only at oxidant-to-protein molar ratios of 10 or more (Table 1). On modification with a 50-fold molar excess of NaOCl, α-helicity was decreased by 44% in lipid-free apo A-I and by 14% in lipid-associated.

ESI-MS analyses of NaOCl- and MPO-modified apo A-I

To analyse the consequences of treatment with OCl^- on Met(SO) formation in lipid-free and lipid-associated apo A-I, the corresponding apo A-I preparations were modified with reagent NaOCl or MPO-generated HOCl at a 6-fold molar oxidant excess as described in the Experimental section. The resulting products were analysed by RP-HPLC. Figure 2 shows representative chromatograms of native, NaOCl- and MPO-modified apo A-I. Native apo A-I was eluted as a single homogeneous peak (Figure 2A) at a retention time of 28.9 min. After modification with either NaOCl or MPO (Figures 2B and 2C), two new peaks (labelled a* and b) occurred. Peak a* eluted at the same retention time as native apo A-I, whereas peak b eluted at 26.8 min. The peaks were collected manually and analysed by ESI-MS under identical conditions to those described in the Experimental section. Native apo A-I exhibited a molecular mass of 28072.6 ± 1.3 Da, in line with that calculated from the amino acid composition. Peaks a* and b revealed molecular masses of 28105.9 ± 1.3 and 28121.7 ± 1.7 Da respectively. The increase in molecular mass of 32 Da (peak a*) and 48 Da (peak b) indicates the addition of two or three oxygen atoms. The corresponding deconvoluted ESI spectra of native apo A-I, peak a* and peak b are shown in Figure 3.

Peptide map and MS analyses of tryptic fragments of native, NaOCl- and MPO-modified apo A-I

To determine which regions within apo A-I are affected by NaOCl- or MPO-derived HOCl, we analysed tryptic digests of native and modified apo A-I. The tryptic fragments were separated by RP-HPLC with UV detection. Representative UV traces of peptide maps of native, NaOCl- and MPO-modified lipid-free apo A-I are shown in Figure 4. To identify tryptic fragments of native apo A-I, RP-HPLC separation was followed by online ESI–MS analyses in a first set of experiments, an analytical tool successfully applied to the identification of tryptic peptides obtained from digests of different proteins [35]. The residue number, sequence and observed molecular masses for the corresponding tryptic peptides of native apo A-I (Figure 4A) are summarized in Table 2. We could identify 23 of the theoretically possible 38 tryptic cleavage products, in line with another study in which tryptic apo A-I peptides were separated by HPLC and subsequently characterized by time-of-flight–selected ion monitoring spectroscopy ('TOF-SIMS') [36]. During the present study T33 and T34 were not cleaved and were detected as one peptide (assigned as 33–34 in Figure 4A). Proteolytic cleavage of T14 and T15 was also not quantitative and some T14–15 adduct was detected. In general we have observed a concentration-dependent loss of individual peptides after modification with

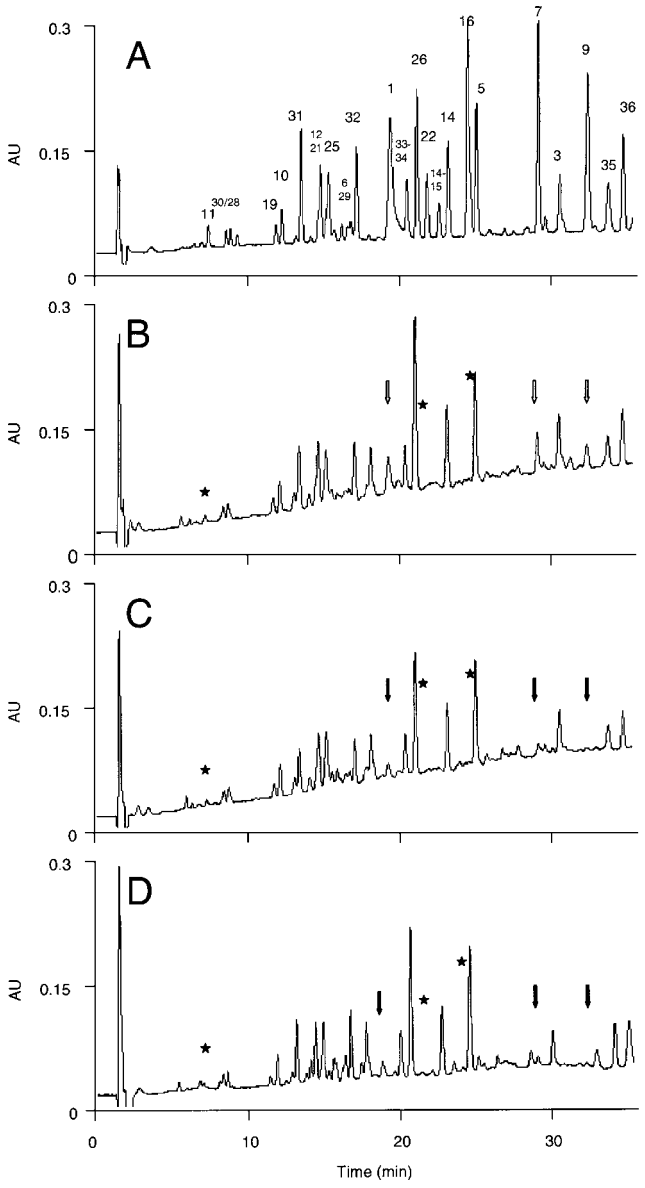

Figure 4 Peptide maps obtained from tryptic digests of native, NaOCl- and MPO-modified lipid-free apo A-I

NaOCl- and MPO-oxidized apo A-I (100 μg) were digested with trypsin (trypsin-to-apo A-I ratio 1:25) as described in the Experimental section. The resulting peptides were analysed by RP-HPLC as described in the Experimental section. Representative UV traces at 215 nm are shown: (**A**) native; (**B**, **C**) NaOCl-modified [5-fold (**B**) and 10-fold (**C**) molar excess of oxidant] and (**D**) MPO-modified apo A-I (10-fold molar excess of oxidant). The peak assignment of the native peptide fragments in (**A**) is identical with that in Table 2. Symbols: ★, original position of Met-containing peptides T11, T16 and T22; open arrows, significantly decreased peak areas of peptides T1, T7 and T9; filled arrows, original positions of lost peptides T1, T7 and T9.

Table 2 Assignment of peptides obtained after tryptic digestion of native apo A-I

Apo A-I (100 μg) was digested with trypsin [ratio of trypsin to apo A-I, 1:25 (w/w)] as described in the Experimental section. The molecular masses of the resulting peptides were obtained by on-line ESI–MS analysis of tryptic fragments under the conditions described in the Experimental section. Peak assignment is identical to that in Figures 4 and 5. Abbreviation: n.d., not detected.

Peak number	Location	Sequence	$[MH]^+$ (amu)
1	1–10	DEPPQSPWDR	1226.8
2	11–12	VK	n.d.
3	13–23	DLATVYVDVLGK	1235.8
4	24–27	DSGR	n.d.
5	28–40	DYVSQFEGSALK	1400.7
6	41–45	QLNLK	615.9
7	46–59	LLDNWDSVTSTFSK	1612.7
8	60–61	LR	n.d.
9	62–77	EQLGPVTQEFWDNLEK	1932.9
10	78–83	ETEGLR	704.8
11	84–88	QEMSK	622.7
12	89–94	NLEEVK	732.7
13	95–96	AK	n.d.
14	97–106	VQPYLDDFQK	1252.8
15	107	K	n.d.
16	108–116	WQEEMELYR	1283.8
17	117–118	QK	n.d.
18	119–123	VEPLR	n.d.
19	124–131	AELQEGAR	873.1
20	132–133	QK	n.d.
21	134–140	LHELQEK	896.7
22	141–149	LSPLGEEMR	1031.7
23	150–151	DR	n.d.
24	152–153	AR	n.d.
25	154–160	AHVDALR	781.8
26	161–171	THLAPYSDELR	1301.8
27	172–173	QR	n.d.
28	174–177	LAAR	430.6
29	178–182	LEALK	574.1
30	183–188	ENGGAR	603.6
31	189–195	LAEYHAK	832.7
32	196–206	ATEHLSTLSEK	1215.8
33	207–208	AK	n.d.
34	209–215	PALEDLR	n.d.
35	216–226	QGLLPVLESFK	1230.7
36	227–238	VSFLSALEEYTK	1386.7
37	239	K	n.d.
38	240–243	LNTQ	n.d.

HOCl. After treatment of apo A-I with a 5-fold molar excess of NaOCl, the Met-containing peptides T11, T16 and T22 disappeared (Figure 5B; their original positions are indicated with filled stars). The peak areas of peptides T1, T7 and T9 were decreased significantly (Figure 4B, open arrows) under the same experimental conditions. At a 10-fold molar excess of reagent NaOCl, a complete loss of peptides T7 and T9 (Figure 4C, filled arrows) and T11, T16 and T22 occurred. The same results with regard to loss of T1, T7, T9, T11, T16 and T22 were obtained after the modification of apo A-I with the $MPO/H_2O_2/Cl^-$ system generating a 10-fold molar oxidant excess (Figure 4D, filled stars and arrows). The same experiments as those described above were performed with lipid-associated apo A-I; almost identical results to those observed with lipid-free apo A-I were obtained (Figure 5).

Online LC–ESI–MS analyses of tryptic digests of NaOCl- and MPO-modified apo A-I were performed under the same conditions as those described above. These experiments verified the loss of peptides T1, T7, T9, T11, T16 and T22 as observed by RP-HPLC with UV detection (Figures 4 and 5). It is well documented that the treatment of apo A-I with H_2O_2 or other oxidants generates Met(SO) groups by a two-electron oxidation step [10–12]. However, all investigators using oxidants weaker than OCl^- reported the oxidation of only two of the three methionine residues in apo A-I (Met^{112} and Met^{148} respectively). In line with these reports we have observed the occurrence of two oxidation products with molecular masses of 1299.6 and 1047.6 a.m.u.

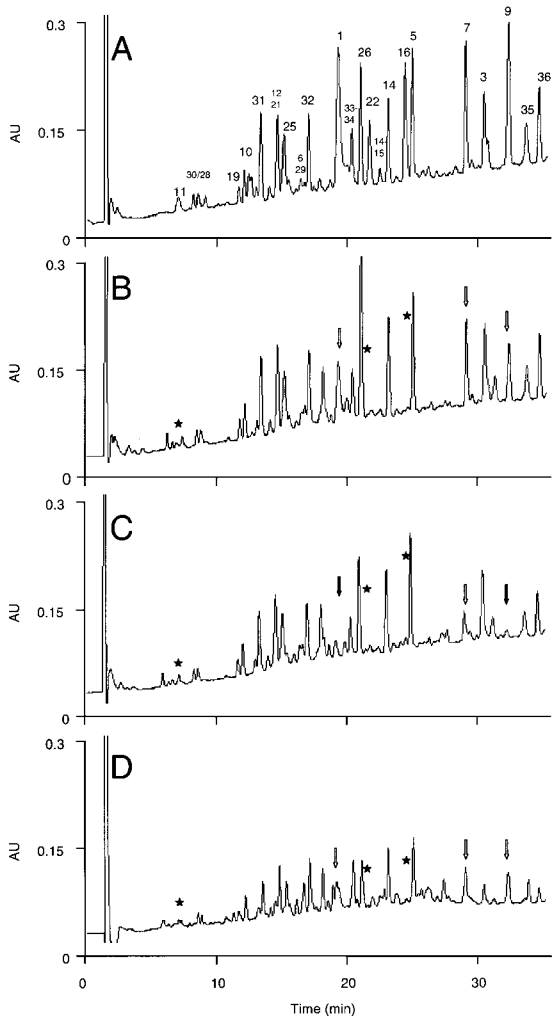

Figure 5 Peptide maps obtained from tryptic digests of native, NaOCl- and MPO-modified lipid-associated apo A-I

Lipid-associated apo A-I was modified as described in the legend to Figure 4. Representative UV traces are shown: (**A**) native; (**B**, **C**) NaOCl-modified [5-fold (**B**) and 10-fold (**C**) molar excess of oxidant] and (**D**) MPO-modified apo A-I (10-fold molar excess of oxidant). The peak assignment of the native peptide fragments in (**A**) is identical with that in Table 2. Symbols: ★, original position of Met-containing peptides T11, T16 and T22; open arrows, significantly decreased peak areas of peptides T1, T7 and T9; filled arrows, original positions of lost peptides T1, T7 and T9.

corresponding to the those of native peptides T16 and T22 plus 16 a.m.u. after treatment with OCl^-. In addition we have detected a new peak with a molecular mass of 638.7 a.m.u., corresponding to the mass of native T11 peptide plus 16 a.m.u.. Because fragments T11, T16 and T22 each contained one methionine residue, the increase in the molecular mass by 16 a.m.u. was most probably due to the formation of Met(SO)[86], Met(SO)[112] and Met(SO)[148]. Oxidized peptides T11, T16 and T22 were not detectable in the tryptic digests of native apo A-I. In addition to the Met(SO)-containing products we have observed the occurrence of two new peaks at 1646.2 a.m.u. [MH^+] (823.6 [$MH^{2+}/2$]) and 1966 a.m.u. [MH^+] (983.5 [$MH^{2+}/2$]). These peaks were not present in native apo A-I peptide maps and resemble the masses of native peptides T7 and T9 plus 33.5 and 33.1 a.m.u., indicating a substitution of Cl for H. The molecular masses of native and modified peptides T7, T9, T11, T16 and T22 and the possible modifications are summarized in Table 3.

Table 3 Molecular masses of HOCl-modified peptides

The molecular masses of peptides T7, T9, T11, T16 and T22 and their corresponding oxidation products were obtained by on-line ESI–MS analyses of the tryptic digests of native and NaOCl-modified apo A-I (100 μg of protein) as described in the Experimental section.

Peak number	Sequence	$[MH]^+$ (amu) Native	Modified	Possible modification
7	LLDNWDSVTSTFSK	1612.7	1646.2	$-H+Cl$
9	EQLGPVTQEFWDNLEK	1932.9	1966.0	$-H+Cl$
11	QEMSK	622.7	638.7	$+O$
16	WQEEMELYR	1283.8	1299.6	$+O$
22	LSPLGEEMR	1031.7	1047.6	$+O$

Fragments T7 and T9 contain amino acids known to be affected by treatment with NaOCl (Trp, Phe and Lys in T7; Pro, Phe, Trp and Lys in T9). To clarify which amino acid(s) is/are attacked by oxidation with NaOCl, the peptides were collected manually during RP-HPLC of tryptic apo A-I maps, oxidized with a 4-fold molar excess of NaOCl and analysed for their amino acid compositions. As can be seen from Table 4, treatment of peptides T7 and T9 with NaOCl resulted in a quantitative loss of Phe residues. The decreased content of Leu in peptide T7 after modification with NaOCl is probably due to the formation of an N-terminal aldehyde product, by reactions similar to those described in [20]. In addition, the contents of Lys and Trp residues were decreased by 20 % and 55 % respectively. Similar observations were made with peptide T9. However, the quantitative loss of Phe residues in T7 and T9 after modification with OCl^- suggests that Phe is a primary target, indicating that the increase of 33.5 and 33.1 a.m.u. is most probably due to the formation of a chlorinated Phe residue. Identical results were obtained with synthetic T7 and T9 peptides (results not shown).

To confirm the formation of chlorinated Phe derivatives, T7, T9 and apo A-I were modified with NaOCl, whereas HDL_3 was modified in parallel by the MPO/H_2O_2/halide system. The reaction mixtures were dried under reduced pressure, hydrolysed in HBr, purified by solid-phase extraction, converted to the corresponding n-propyl-*N*-heptafluorobutyryl derivatives and analysed by NICI–MS, EI–MS and selected-reaction-monitoring GLC–MS. In Figure 6 the ion chromatograms at m/z 436 ($[M-H]^-$, see below) of an authentic 4-Cl-Phe standard (Figure 6A) and HBr hydrolysates of NaOCl-modified apo A-I (Figure 6B) and MPO-modified HDL_3 (Figure 6C) are displayed. To exclude the possibility of an artifact of chlorination during sample processing, apo A-I was hydrolysed in HBr but in the presence of a 10-fold molar excess of Cl^- ions (Figure 6D; the total ion intensity of the trace is approx. 1/20 that in Figures 6A–6C). The derivative of the 4-Cl-Phe standard eluted as a single, homogeneous peak at a retention time of 4.98 min, which was also present in NaOCl-modified apo A-I and in MPO-modified HDL_3. In addition we observed one peak at 4.90 min in NaOCl-modified apo A-I (but not in MPO-modified HDL_3; Figures 6B and 6C). None of these peaks was observed in hydrolysates obtained from native apo A-I or HDL_3. When apo A-I was hydrolysed in the presence of Cl^- we observed traces of 4-Cl-Phe, corresponding to approx. 0.12 % of the peak area seen

Table 4 Amino acid compositions of NaOCl-oxidized peptides T7 and T9

Peptides T7 and T9 were collected manually from the tryptic digest and oxidized with a 4-fold molar excess of NaOCl as described in the Experimental section. Amino acid analysis was performed on hydrolysates prepared under vacuum in 6 M HCl. Trp residues were measured fluorimetrically [31]. Results are means for two independent experiments and are expressed as percentages of the amino acids present in the native peptides.

NaOCl-oxidized T7		NaOCl-oxidized T9	
Amino acid	Content (% of native)	Amino acid	Content (% of native)
D + N	100	D + N	100
S	104	E + Q	75
T	94	G	99
V	95	P	99
K	81	V	99
L	63	K	78
F	0	L	85
W	45	F	0
		W	56

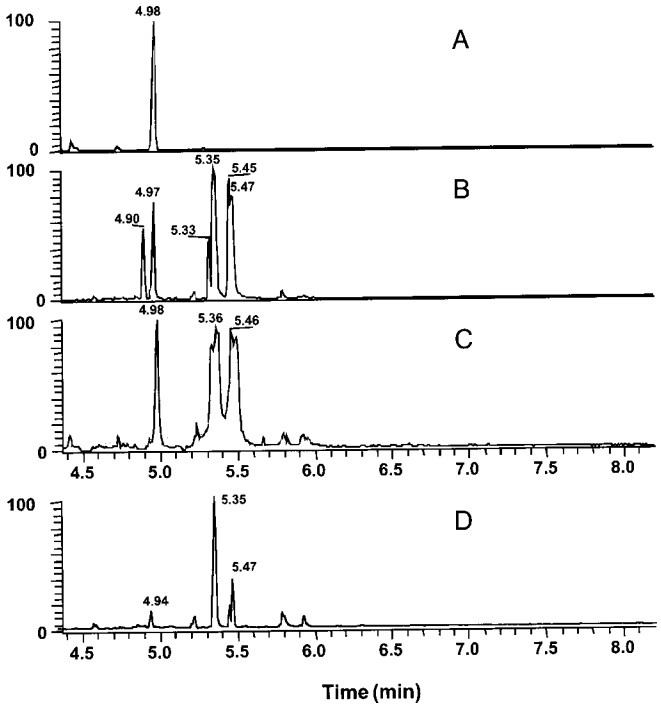

Figure 6 Detection of 4-Cl-Phe in HBr hydrolysates obtained from OCl^--modified apo A-I and HDL_3 by selected-ion-monitoring NICI GLC–MS analysis

Ion chromatograms at *m/z* 436 of (**A**) an authentic 4-Cl-Phe standard and (**B**–**D**) hydrolysate of NaOCl-modified apo A-I (**B**), MPO-modified HDL_3 (**C**) and native apo A-I hydrolysed in the presence of Cl^- (**D**). Samples were analysed as their n-propylheptafluorobutyryl derivatives with NICI-MS as described in the Experimental section. The peak cluster that was eluted between 5.30 and 5.50 min was not identified.

in NaOCl- or MPO-modified samples, excluding the possibility that the 4-Cl-Phe formation was an artifact due to the chlorination of apo A-I. We did not attempt to identify the peak cluster that was eluted between 5.30 and 5.50 min (Figures 6B–6D) because the mass spectra were different from the monochlorinated Phe derivatives.

The NICI mass spectra of the authentic standard, and the peaks that were eluted at 4.90 and 4.97 min (Figures 6B and 6C) are shown in Figures 7(A–C) respectively. The NICI mass spectrum of 4-Cl-Phe is characterized by an $[M-H]^-$ ion (*m/z* 436), three successive HF abstractions (*m/z* 417, 397 and 377) and an ion at *m/z* 338 resulting from $C_3H_7O^{\cdot}$ abstraction from the *m/z* 377 fragment. The isotope distribution is indicative of a monochlorinated compound. Identical mass spectra were obtained for the peaks at retention times of 4.90 (Figure 7B) and 4.97 min (Figure 7C), indicating that the peak at 4.97 min (present in modified apo A-I and HDL_3) corresponds to 4-Cl-Phe, whereas the peak at 4.90 min most probably represents the 2- or 3-Cl-Phe derivative. The NICI spectra in Figure 7 confirm the findings of Phe loss in T7 and T9 and indicate that the modification of apo A-I with NaOCl or the modification of HDL_3 with MPO under the conditions employed during the present study results in the formation of chlorinated Phe residues in apo A-I. During EI^+ experiments an abundant ion resulted from the α-cleavage of 4-Cl-Phe, giving rise to an *m/z* 350 fragment. This fragment was used as parent ion in MS–MS experiments and gave rise to prominent secondary-ion formation at *m/z* 332, 312 and 213 in identical manners for the authentic 4-Cl-Phe standard, T7, T9 and hydrolysates obtained from NaOCl-modified apo A-I and MPO-modified HDL (results not shown).

The effects of increasing NaOCl amounts on modification of apo A-I in discrete regions (as observed by changes in the peptide maps of apo A-I preparations) are summarized in Figure 8. Affected, disappearing peptides are marked with an arrow, whereas peptides having markedly decreased peak areas are shown in parentheses. On the modification of apo A-I with increasing NaOCl concentrations or in the presence of the $MPO/H_2O_2/Cl^-$ system, the following alterations in the peptide maps occurred: at a 5-fold molar excess of NaOCl, peaks corresponding to T11, T16 and T22 were lost (Figure 8A), followed by the disappearance of peptides T1, T7 and T9 at an oxidant-to-apoprotein molar ratio of 10:1 (Figure 8B). The same modification pattern occurred when the $MPO/H_2O_2/Cl^-$ system generating a 10-fold molar excess of oxidant was used (Figure 8D). At a 25-fold molar excess of NaOCl a marked decrease in the peak areas of peptide fragments T31 and T32 in comparison with other C-terminal fragments, i.e. T35 and T36, was observed (Figure 8C). At NaOCl-to-apo A-I ratios of 25:1 or more the peak area of all peptides was significantly decreased, probably as a result of intermolecular and/or intramolecular cross-links in the apo A-I molecule.

DISCUSSION

In the present study we characterized two distinct oxidized forms of apo A-I, namely apo $A\text{-}I_{+32}$ and apo $A\text{-}I_{+48}$, with molecular masses suggesting the addition of two or three oxygen atoms to the native protein after modification with physiologically occurring concentrations of HOCl [37]. Previous studies have shown that only two of the three Met residues (Met^{112} and Met^{148}) in apo A-I are susceptible to artificial oxidation by H_2O_2 [10–12]. Recently, Garner et al. [12] have reported the formation of apo $A\text{-}I_{+16}$ and apo $A\text{-}I_{+32}$ after the oxidation of HDL with 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), a water-soluble radical generator. On the basis of these findings, the authors suggested that methionine oxidation in HDL-associated apo A-I can occur in a stepwise manner, rather than

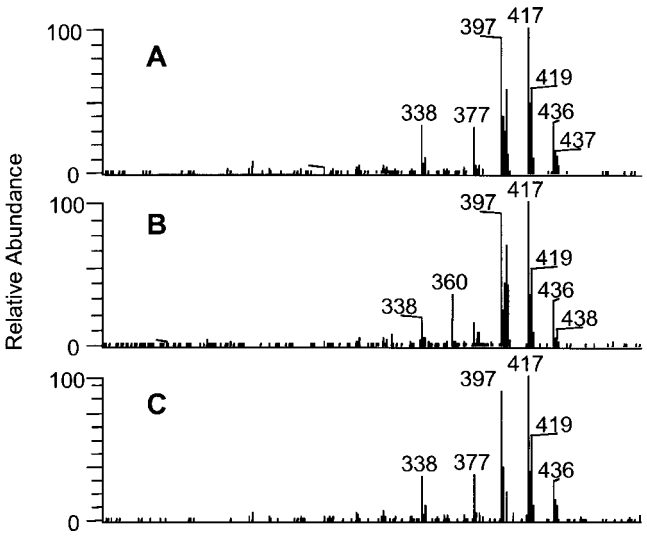

Figure 7 NICI mass spectra

NICI mass spectra of the n-propylheptafluorobutyryl derivatives of (**A**) an authentic 4-Cl-Phe standard (4.98 min in Figure 6A), (**B**) the peak that was eluted at 4.90 min in Figure 6(B) and (**C**) the peak that was co-eluted with the authentic standard in Figures 6(B) and 6(C) respectively (at 4.97 min). The fragment *m*/*z* 436 corresponds to $[M-H]^-$ of the n-propylheptafluorobutyryl derivative of 4-Cl-Phe. Three successive HF abstractions gave rise to the fragments at *m*/*z* 417, 397 and 377. The ion at *m*/*z* 338 results from $C_3H_7O^{\bullet}$ abstraction from the *m*/*z* 377 fragment.

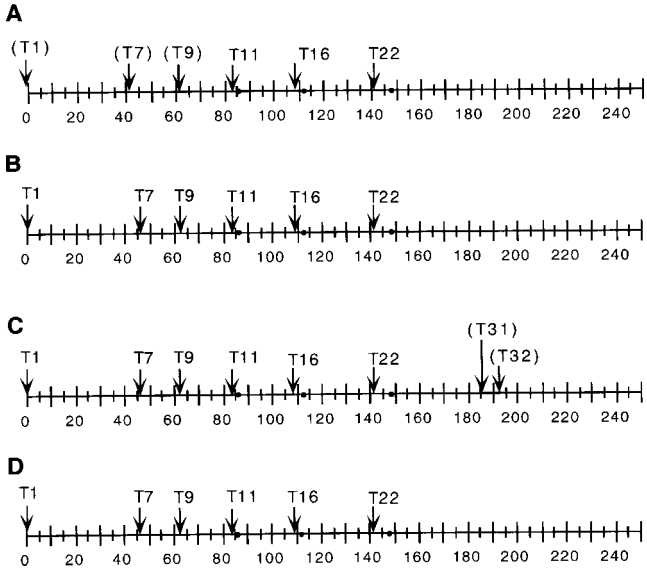

Figure 8 Linear sequence maps of native, NaOCl- and MPO-modified apo A-I

Linear sequence maps of apo A-I summarize the observed disappearance of peptide fragments after modification with NaOCl [molar excess of oxidant 5 (**A**), 10 (**B**) and 25 (**C**)] and MPO oxidation [molar excess of oxidant 10 (**D**)]. The positions of Met^{86}, Met^{112} and Met^{148} are indicated by filled circles. Peptide fragments that were lost during the oxidation are indicated with arrows; peptide fragments that exhibited markedly decreased peak areas in the UV traces are shown in parentheses.

the 'none or two' methionine-oxidation mechanism proposed previously [11]. After modification of apo A-I with OCl^- we have three lines of evidence that all three methionine residues are susceptible to oxidation *in vitro*: (1) RP-HPLC analysis and subsequent off-line ESI–MS indicated the formation of two oxidized forms of apo A-I, namely apo A-I_{+32} and apo A-I_{+48}; (2) the three Met-containing peptides (T11, T16 and T22) disappeared in the UV and total-ion-current traces of the peptide maps; and (3) RP-HPLC and on-line ESI–MS analyses indicated the formation of oxidation products of the Met-containing peptides T11, T16 and T22, each having a molecular mass 16 a.m.u. higher than the non-oxidized peptide. The disappearance of the Met-containing peptide T11 after OCl^- treatment, with the concomitant appearance of an oxidation product (with a mass of an additional 16 a.m.u.), indicates the formation of Met(SO) at residue Met^{86}. We did not detect a specific oxidized form of apo A-I owing to the oxidation of one single Met residue, which can be ascribed to the use of different oxidation systems from those of other investigators (OCl^- compared with AAPH or H_2O_2 in [12] and [13]). As can be seen from the UV traces of the peptide maps, Met-free peptides are not lost until all Met-containing peptides are oxidized. This observation further supports the anti-oxidative properties of Met in proteins and peptides, as discussed by others [12,38,39]. Recently it has been shown that oxidized Met^{112} and Met^{148} in lipid-free as well as in lipid-associated apo A-I can be reduced to Met *in vitro* by peptide Met(SO) reductase [40], suggesting that the enzymic reduction of oxidized apo A-I could restore its biological activity.

We could further demonstrate that the modification of apo A-I with OCl^- results in a concentration-dependent loss of α-helicity in lipid-free (and to a smaller extent in lipid-associated) apo A-I. It has been reported that the oxidation of Met^{112} and Met^{148} induces changes in the secondary structure of apo A-I, resulting in a marked decrease in α-helicity [7]. In line with these findings, changes in the secondary structure occurring at low molar ratios of HOCl to apo A-I (5:1 for lipid-free and 10:1 for lipid-associated apo A-I) are most probably due to the formation of the corresponding sulphoxides. One might expect that methionine oxidation induces structural changes in the entire protein, affecting the susceptibility of distinct regions of apo A-I to further attack by HOCl. We have noticed that the oxidation of Met^{86}, Met^{112} and Met^{148} precedes modification of peptides T1, T7 and T9 by OCl^- in the N-terminal region of apo A-I. This is in line with findings by Roberts et al. [7], who observed altered protease susceptibility of apo A-I after oxidation of Met^{112} and Met^{148} with newly occurring cleavage sites in the N-terminal part of the protein. Interestingly, two of the new cleavage sites described in [7] are located in T7 or adjacent to T9.

After modification of T7 and T9 by OCl^- we have identified products with molecular masses 33.5 and 33.1 a.m.u. greater than those of the native peptides. Both peptides contain amino acids that are affected by HOCl oxidation (Phe, Trp and Lys [17,22,29,41]). As confirmed by amino acid analysis of native and oxidized peptides T7 and T9, Phe is apparently the primary target of modification with HOCl in these fragments, at least at low oxidant concentrations. The quantitative loss of Phe from both peptides after modification with HOCl (as determined by amino acid analysis) is most probably a reflection of the formation of 4-Cl-Phe and a second chlorinated isomer (either the 2-chloro or the 3-chloro derivative). These findings were confirmed by GLC–NICI–MS analysis of hydrolysates obtained from NaOCl-modified peptides and apo A-I. The reason for the absence of the chlorinated Phe-isomer from MPO-modified HDL_3 is currently not clear. However, these observations are in line with findings that *N*-acetylphenylalanine undergoes ring chlorination on treatment with HOCl, NO_2^-/HOCl or nitryl chloride [41]. On the basis of isomer distribution (preferential formation of the *p*- and *o*- derivatives) it was suggested [41] that Phe chlorination by HOCl occurs by an ionic rather than by a

radical-mediated process. It remains to be elucidated whether or not one of the 4-Cl-Phe isomers represents a useful marker molecule for detecting MPO-modified proteins *in vivo*. The observation that NaOCl-modified amino acids decompose on RP columns [42] (in addition to secondary degradation reactions of chlorination products of Phe) could explain the presence of relatively small concentrations of oxidation products in comparison with the native peptides. The formation of a chloramine at the α-amino group of Asp^1 in peptide T1 (by either NaOCl or MPO) and the subsequent generation of an aldehyde by similar pathways to those proposed in [20] would provide a plausible explanation for the loss of peptide T1 observed during the present study.

Several groups have suggested that specific amino acid sequences participate in HDL-mediated cholesterol efflux [4,5,43,44]. In addition it has been suggested that the presence of amphipathic α-helices with high lipid-binding affinity determines the ability of apoproteins to participate in the membrane microsolubilization of cellular phospholipids and cholesterol [45]. Therefore the structural integrity of apo A-I is an important factor in determining its ability to participate in reverse cholesterol transport. A major question arising from the present study was whether the relatively moderate apo A-I modifications are sufficient to interfere with the capacity to promote cellular cholesterol efflux. We now have evidence that the modification of DPPC-associated apo A-I by HOCl results in impaired cholesterol efflux from J774 macrophages even at low modification rates [46] with no detectable formation of cross-linked, high-molecular-mass apo A-I. This could result from the modification of T9, T11, T16 and T22, peptides overlapping with specific amino acid sequences contributing to cellular cholesterol efflux [4,5,43,44]. The initial efflux of cellular unesterified cholesterol to HDL occurs by one of two independent mechanisms [47,48], depending on the degree of apo A-I lipidation. In its fully lipidated state cellular cholesterol is removed by an aqueous diffusion process [49], whereas cholesterol efflux promoted by lipid-free or lipid-poor apo A-I is mediated by a membrane microsolubilization process of phospholipids together with unesterified cholesterol [50]. In conjunction with the decreased α-helical content of modified lipid-free apo A-I, modification by OCl^- could affect both of these mechanisms mediating cellular cholesterol removal.

We thank Dr Tony Kettle (Christchurch, New Zealand) and Dr Ch. Obinger (Vienna, Austria) for MPO samples, and Dr C. Siethoff from Spectronex (Basel, Switzerland) for deconvolution of the ESI spectra. This work was supported by grants P12000 MED (W. S.) and P11276 MED (E.M.) from the Austrian Research Foundation (FWF). We acknowledge financial support by the Franz-Lanyar Stiftung and the Austrian National-Bank (projects 8127 to W. S. and 7434 to E. M.).

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Received 17 September 1999/11 November 1999; accepted 2 December 1999