Supplementary data

2-Chlorohexadecanal, derived from hypochlorite-modified high-density lipoprotein-associated plasmalogen is a natural inhibitor of endothelial nitric oxide biosynthesis

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MATERIALS AND METHODS

Lipoproteins

Low density lipoprotein (LDL, \(d = 1.035\) to \(1.065 \text{ g/ml}\)) and HDL (\(d = 1.125\) to \(1.21 \text{ g/ml}\)) was prepared by discontinuous density ultracentrifugation from plasma obtained from normolipidemic, healthy blood donors.\(^8,26\) HOCl-modification of lipoproteins (at indicated oxidant : lipoprotein molar ratios) and characterization of the modified lipoprotein particles was performed as described.\(^8,13,26\) HOCl-modified lipoproteins were stored at \(4^\circ\text{C}\) and used within 24 h after exposure to the oxidant.

Preparation of lipid micro-emulsions and acid hydrolysis of lipoprotein-associated lipids

Lipids were extracted from native or modified lipoproteins as described.\(^26\) Briefly, HCl was added to the lipoprotein preparations to a final concentration of 10 mM, and the lipids were extracted using chloroform/methanol (2:1, v/v). The chloroform phase was removed and evaporated under nitrogen, and the lipids were resuspended in tris-buffered saline (0.01 M Tris/HCl, 0.001 M EDTA, 0.15 M NaCl, pH 7.4), sonicated and extruded through 0.1 \(\mu\text{m}\) polycarbonate membranes to form microemulsions. Where mentioned, lipid micro-emulsions obtained from native HDL were modified with HOCl according to an oxidant:lipoprotein molar ratio of 50:1 (calculated on the total cholesterol (TC) content of HDL as measured by the CHOD-PAP method, Roche Applied Science).
For preparation of plasmalogen-free lipid microemulsions, lipids were extracted as described above. The chloroform phase was removed and treated with HCl (addition of 100 µl 4 M HCl to 1 ml of chloroform) and vortexed for 4 min. Subsequently the mixture was washed three times with water. The chloroform phase was then evaporated under nitrogen, and the lipids were resuspended in Tris-buffered saline as described above.

Synthesis, derivatization and quantitation of 2-ClHDA by gas chromatography-mass spectrometry (GC-MS)

Synthesis: 2-ClHDA was synthesized and purified as described with modifications. Briefly, 40 mg MnCl₂ were dissolved in 1 ml of methanol, followed by the addition of 40 mg MnO₂. The mixture was stirred and heated to 40°C. Ten mg of hexadecanal dimethyl acetal (dissolved in 1 ml acetonitrile) and 240 µl of trimethylsilylchlorid were added to form a MnO₂-trimethylchlorosilane-chlorinating reagent. After 6 h, the solution was cooled to 25°C, diluted with 5 ml of hexane and extracted with 2 ml aqueous NaOH (1.5 %, w/v). After washing with water, the hexane layer, containing 2-ClHDA dimethyl acetal, was dried under a stream of nitrogen. The resulting colourless oil was dissolved in 100 µl CH₂Cl₂. After addition of 100 µl trifluoroacetic acid the vial was closed, heated for 1 h at 75°C, and after that the solvent again was removed by nitrogen.

Derivatization: 2-ClHDA as well as lipid extracts from native and modified lipoproteins/lipids, were dissolved in 100 µl ethanol and 100 µl of a solution of pentafluorobenzyl (PFB) hydroxylamine in ethanol (6 mg/ml, w/v) were added. After 1 h at 25°C, 1 ml of distilled water was added, and the PFB oxime derivative was extracted with cyclohexane/diethyl ether (4:1, v/v) and dried under nitrogen. The samples were redissolved in 100 µl petroleum ether, transferred to autosampler vials and stored at –20°C until GC/MS analysis.
**Quantitation by GC-MS analysis:** Samples were analyzed on a SGE BPX5 capillary column (15 m, I.D: 0.25 mm inner diameter, 0.25 µm methyl silicone film coating) using a Finnigan Trace GC-MS (helium was used as carrier gas, 2 ml/min). Injector temperature was set to 220°C and ion source temperature was 180°C. The oven temperature was maintained at 80°C for 1 min, increased at a rate of 30°C/min to 300°C and held at 300°C for additional 5 min. All spectra were monitored in negative ion chemical ionization (methane as reagent gas), either in full scan mode or using selected ion monitoring at m/z = 196 (base peak) and m/z = 288/290 (characteristic isotopic distribution of chlorine in the corresponding C16:0 fatty aldehyde residue). Semiquantitative analysis of 2-ClHDA formation was performed by spiking native lipoproteins as well as lipoproteins modified by HOCl (added as reagent or generated by the MPO-hydrogen peroxide-chloride system of activated phagocytes) with different concentrations of synthetic 2-ClHDA and comparison of the corresponding peak areas.

**Cell culture experiments**

HUVECs were isolated and cultured as described. At passages 1 or 2 the cells were incubated with lipoproteins, lipid micro-emulsions or 2-ClHDA in culture medium up to 18 h. The indicated amounts of lipoproteins or lipids used in the experiments were based upon the TC content. Cell viability and apoptosis, incorporation of [14C]-labeled amino acids, and protein determination were performed as described.

**Human neutrophils**

Neutrophils were isolated from blood of healthy human donors by buoyant density centrifugation as described using Hank’s balanced salt solution (Mg²⁺-, Ca²⁺-, phenol- and bicarbonate-free, pH 7.2) supplemented with 100 µM diethylentriamine pentaacetic acid. Cells (1 x 10⁶) were
stimulated with phorbol-12-myristate-13-acetate (200 nM) in the presence of HDL (30 µg TC/ml) in the absence or presence of 1 mM sodium azide or methionine for 60 min at 37°C. After centrifugation of the cells, lipoprotein-associated 2-ClHDA was extracted with chloroform/methanol and analyzed.

**eNOS activation assays**

Preincubation of HUVECs with lipoproteins, lipid micro-emulsions or 2-ClHDA was performed in culture medium (containing 15 % FCS and 5 % human serum) up to 18 h. Stimulation of cells with ionomycin (2 µM, 15 min) or thrombin (1 U/ml, 15 min) was performed in the absence of serum and lipoproteins, lipid micro-emulsions or 2-ClHDA. Ionomycin and 2-ClHDA were dissolved in DMSO. The final concentration of DMSO during experimental incubations did not exceed 0.1 %. Control cells received the same volume of vehicle. eNOS activation was assessed in whole cells by measuring [³H]L-arginine conversion to [³H]L-citrulline which is formed stochiometrically with NO.

For cGMP measurement performed by RIA (Amersham) the cells were incubated and stimulated for 15 min under the same conditions as described above. The reaction was stopped with 96 % ethanol. After evaporation of the solvent, 0.3 ml of buffer (50 mM Tris, 4 mM EDTA, pH 7.5) were applied. The cGMP content of 100 µl of cellular extract was measured by RIA following the instructions of the manufacturer. The intracellular cGMP concentration was expressed in pmol/mg cell protein. The agonist-induced cGMP production was determined from the difference of cGMP content in ionomycin-stimulated cells and the corresponding unstimulated cells. The agonist-induced L-citrulline formation in HUVECs was completely inhibited when cells were preincubated with 1 mM of the eNOS inhibitor L-nitroarginine-methylester 30 min before stimulation.
[\text{\textsuperscript{14}}C]L-Citrulline synthesis from cell lysates was estimated after preincubation of HUVECs with lipoproteins (18 h, 90 µg TC/ml) as described.

**Northern blot analysis and mRNA stability**

For Northern blot analysis HUVEC monolayers were incubated (8 h) in the absence or presence of HDL or HOCl-HDL (oxidant:lipoprotein molar ratio of 50:1, 90 µg TC/ml). To measure eNOS mRNA stability, confluent HUVECs were incubated for 18 h in the absence or presence of 10 µM 2-ClHDA in media containing 10 µg/ml 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB). Cellular RNA was isolated and Northern blot analysis was performed using a specific eNOS cDNA probe (Alexis Corporation). The blot was then stripped and reprobed with a human GAPDH cDNA fragment (Clontech). Alternatively, the 28S rRNA band was used as loading control.

**Western blotting**

HUVEC monolayers were incubated for 18 h in the absence or presence of lipoproteins, lipid micro-emulsions, or 2-ClHDA. In some experiments DRB (10 µg/ml) or cycloheximide (5 µg/ml) was added in parallel. Cells were scraped and eNOS was detected by Western blotting experiments using monoclonal anti-human eNOS IgG. The β-actin band was taken as indicator for equal protein loading.

**Immunofluorescence microscopy**

HUVEC monolayers were incubated for 18 h in the absence or presence of lipoproteins or 2-ClHDA. Subsequently, the cells were fixed for 20 min with paraformaldehyde in PBS (3.5 % v/v), washed with PBS, and incubated with 5 % goat serum in PBS containing 0.6 % Triton X-
100 for permeabilization. Cells were then incubated for 2 h with a monoclonal anti-human eNOS IgG which was followed by a 2 h incubation with Alexa 488-labeled secondary antibodies (Molecular Probes). Cells were visualized with a laser scanning confocal microscope (Zeiss, Axiovert 135 M).

References


Figure I

*Negative ion chemical ionization GC-MS analysis of 2-ClHDA.*

2-ClHDA was converted to the corresponding PFB oxime derivative and subjected to GC-MS analysis as described in Methods. Selected ion monitoring (m/z = 288) of PFB oxime derivatives of the indicated samples is shown. The corresponding mass spectra of the PFB oximes of 2-ClHDA from synthetic 2-ClHDA and 2-ClHDA generated in HDL after HOCl-treatment (oxidant:lipoprotein molar ratio of 100:1) are shown. For experimental details see Methods.
Figure II

*Full scan spectra of 2-ClHDA in GC-NICI-MS analysis*

The corresponding mass spectrum of the PFB oximes from synthetic 2-ClHDA is shown. For experimental details see Methods. Prominent fragmentation peaks occurred at m/z 196 (base peak) and at m/z 288/290. The characteristic isotopic distribution of chlorine in the corresponding C16:0 fatty aldehyde residue is shown in the molecule structure (insert).
Figure III

Full scan spectra of 2-ClHDA in GC-NICI-MS analysis

The corresponding mass spectrum of the PFB oximes from 2-ClHDA generated in HDL after HOCl-treatment (oxidant:lipoprotein molar ratio of 100:1) is shown. For experimental details see Methods. Prominent fragmentation peaks occurred at m/z 196 (base peak) and at m/z 288/290.