2-Chlorohexadecanal Derived From Hypochlorite-Modified High-Density Lipoprotein–Associated Plasmalogen Is a Natural Inhibitor of Endothelial Nitric Oxide Biosynthesis

Gunther Marsche, Regine Heller, Günter Fauler, Alenka Kovacevic, Alexander Nuszkowski, Wolfgang Graier, Wolfgang Sattler, Ernst Malle

**Objective**—Myeloperoxidase, a heme enzyme that is present and active in human atherosclerotic lesions, provides a source for the generation of proinflammatory chlorinated reactants contributing to endothelial dysfunction. Modification of high-density lipoprotein (HDL) by hypochlorous acid/hypochlorite (HOCl/OCl⁻)—generated in vivo by the myeloperoxidase-hydrogen peroxide-chloride system of activated phagocytes—forms a proatherogenic lipoprotein particle that binds to and is internalized by endothelial cells.

**Methods and Results**—Here we show that HDL, modified with physiologically relevant HOCl concentrations, attenuates the expression and activity of vasculoprotective endothelial nitric oxide synthase. HOCl-HDL promotes dislocation of endothelial nitric oxide synthase from the plasma membrane and perinuclear location of human umbilical venous endothelial cells. We could identify 2-chlorohexadecanal as the active component mediating this inhibitory activity. This chlorinated fatty aldehyde is formed during HOCl-mediated oxidative cleavage of HDL-associated plasmalogen.

**Conclusion**—2-Chlorohexadecanal, produced by the myeloperoxidase-hydrogen peroxide-chloride system of activated phagocytes may act as a mediator of vascular injury associated with ischemia-reperfusion injury, glomerulosclerosis, and atherosclerosis. (Arterioscler Thromb Vascul Biol. 2004;24:1-5.)

**Key Words:** myeloperoxidase • 2-chlorinated fatty aldehyde • atherosclerosis • modified lipids • glomerulosclerosis • neutrophils

Animal experimentation and clinical studies have provided convincing evidence that the known risk factors for cardiovascular disease can elicit a localized inflammatory response in the vasculature. The changes are most pronounced in endothelial cells and include oxidative stress, increased activation of endothelial signaling pathways, and the consequent adhesion, activation, and degranulation of leukocytes. The myeloperoxidase (MPO)-hydrogen peroxide-chloride system of stimulated leukocytes, primarily neutrophils, generates hypochlorous acid/hypochlorite (HOCl/OCl⁻), a potent bacterial oxidant in vivo. MPO and HOCl are emerging as critical modulators of vascular injury by promoting inflammatory arterial pathology and subsequent formation of mature plaques. MPO is present and active in human lesion material. HOCl reacts with a wide range of biological substrates, including antioxidants, amines, sulfides, nucleotides, DNA, lipids, and (lipo)proteins. HOCl-modified (lipo)proteins are present in human ²⁻⁸ and rabbit lesions, ⁹,¹⁰ and disease stage-dependent accumulation of HOCl-modified (lipo)proteins has been reported. Elevated levels of plasma high-density lipoprotein (HDL) protect against atherosclerotic vascular disease. A broad spectrum of potent antioxidant and anti-inflammatory activities has been ascribed to native HDL. ¹¹,¹² However, oxidation/modification by HOCl alters the physicochemical and metabolic properties of anti-atherogenic HDL. ¹³⁻¹⁵ In vivo, apolipoprotein A-I, the major apolipoprotein of HDL, represents a selective target for MPO-catalyzed oxidation; a process apparently facilitated by MPO binding to apolipoprotein A-I. ¹⁶ Furthermore, significantly increased levels of 3-chlorotyrosine, a protein modification specific for the MPO-hydrogen peroxide-chloride-catalyzed oxidation, ¹⁷ were detected in association with apolipoprotein A-I recovered from serum of patients with cardiovascular disease. ¹⁶ In situ experiments revealed colocalization of specific epitopes derived from HOCl with apolipoprotein A-I in human atherosclerotic lesions and on endothelial cells lining the blood vessel. ⁸ MPO levels serve as a strong predictor of endothelial dysfunction in humans,¹⁸ and MPO colocalizes with HOCl-modified epitopes/(lipo)proteins on endothelial cells.
Native and modified lipoproteins have been reported to affect levels of nitric oxide (NO),19–24 an ubiquitous signaling molecule modulating a plethora of physiological responses including the propagation of a number of anti-atherogenic effects in the endothelium.25 Here, we show that treatment of human umbilical venous endothelial cells (HUVECs) with HDL modified by HOCl impairs expression and activity of endothelial nitric oxide synthase (eNOS). The active component mediating this effect was found to be 2-chlorohexadecanal (2-ClHDA) formed via HOCl-mediated oxidative attack of HDL-associated plasmalogen.

Materials and Methods
A detailed Materials and Methods section on lipoproteins,8,13,26 preparation of lipid micro-emulsions and acid hydrolysis of lipoprotein-associated lipids,26,27 synthesis, derivatization, and quantitation of 2-ClHDA by gas chromatography–mass spectrometry,28–30 cell culture experiments,24 neutrophil stimulation,9,27 eNOS activation assays,24,31 Northern blot analysis and mRNA stability,24 Western blotting,24 and immunofluorescence is available online at http://atvb.ahajournals.org.

Results and Discussion
The effect of HDL to modulate NO release from endothelial cells has been investigated by several groups.32 Here, we show that HDL modified with HOCl concentrations that are easily achieved under acute inflammation13,33 caused a marked, sustained, concentration-dependent, and time-dependent decrease of NO production in stimulated HUVECs as measured by the conversion of L-arginine to L-citrulline (Figure 1A to 1C). To confirm the functional significance of these findings, we assessed the effects of HOCl-HDL on the production of cGMP, a second messenger formed via activation of soluble guanylate cyclase by NO. As expected, HOCl-HDL caused a marked reduction of cellular cGMP production (Figure 1A and 1B). Of note, HOCl-HDL did not affect endothelial cell viability or alter cell growth behavior as determined by trypan blue exclusion and incorporation of 14C-labeled amino acids. Furthermore, no evidence of endothelial cell apoptosis was seen under the experimental conditions described (data not shown).

The efficacy of NO biosynthesis critically depends on the correct compartmentalization of eNOS in specific intracellular membrane domains, i.e., Golgi complex and plasmalemmal caveolae.34,35 We previously reported that low-density lipoprotein (LDL) modified by reagent HOCl inhibits NO synthesis in HUVECs via an intracellular dislocalization of eNOS without altering eNOS protein expression and without altering myristoylation or palmitoylation of the enzyme.24 Here, we show that coincubation of HUVECs with HOCl-HDL also leads to changes of intracellular eNOS distribution including translocation from the plasma membrane and disintegration of the perinuclear location (Figure 2A). But in contrast to HOCl-LDL, HOCl-HDL further decreased eNOS mRNA levels (Figure 2B and 2C). HOCl-HDL also induced a marked downregulation of eNOS protein expression when cells were incubated in the presence of DRB, a transcription inhibitor (Figure 2D). When cells were incubated with cycloheximide, an inhibitor of protein synthesis, HOCl-HDL had no significant effect indicating that a post-translational destabilization of eNOS is not involved; thus, it is conceivable that downregulation of eNOS is a post-transcriptional event caused by decreased stability of eNOS mRNA. In line with these observations, NO biosynthesis in cell lysates in the presence of saturating concentrations of substrate and cofactors31 was also decreased (Figure 1A and 1B).
Plasmalogens, localized in the plasma membranes of mammalian cells and present in lipoproteins, are considered to be antioxidants because the vinyl ether bond is susceptible to attack by oxidants. Recent findings revealed that the vinyl ether bonds of plasmalogens are highly accessible molecular targets of the membrane-permeable, reactive chlorinating species generated by the MPO-hydrogen peroxide-chloride system; as a result, lysophosphatidylcholine and α-chlorinated fatty aldehydes are formed. Thukkani et al have shown that 2-CHDA is formed as the main α-chloro fatty aldehyde from cell-associated plasmalogens. In another report, these authors demonstrated that 2-CHDA is also generated when LDL was coincubated with stimulated monocytes. Therefore, we first tested whether HOCl (added as reagent) targets HDL-associated plasmalogens, leading to 2-CHDA formation. For external quantification, 2-CHDA was synthesized and characterized by gas chromatography–mass spectrometry analysis. Analysis of PFB oximes from synthetic 2-CHDA and 2-CHDA isolated from lipid extracts from HOCl-HDL (Figure I, available online at http://atvb.ahajournals.org) showed identical results regarding retention time of 7.25 minutes and 7.15 minutes (an expected C16:0 isofrom), as well as full scan spectra (Figures II and III, available online at http://atvb.ahajournals.org). Prominent fragmentation peaks occurred at m/z 196 and m/z 288/290. Additional peaks from lipid extracts obtained from HOCl-HDL could be identified as dichlorinated hexadecanol (7.42 minutes), monochlorinated C18:1 fatty aldehyde (7.70 minutes), and a monochlorinated C18:0 fatty aldehyde (7.77 minutes). 2-CHDA could not be detected in native HDL (Figure I) or in plasmalogens (data not shown). When plasmalogen-enriched HDL was subjected to HOCl-treatment, 2-CHDA concentrations increased several-fold compared with HOCl-HDL (data not shown).

The plasmalogen content in HDL is \( \approx 100 \) mmol/mg TC. After HOCl-modification of HDL (oxidant:lipoprotein molar ratio of 100:1) \( \approx 80\% \) of the total plasmalogen fraction contributes to 2-CHDA formation (Figure 4A). HOCl-modification of HDL (oxidant:lipoprotein molar ratio of 25:1 and 100:1; 90 \( \mu \)g TC/mL) resulted in the generation of \( \approx 4 \) to 6.5 \( \mu \)mol/L 2-CHDA. These levels are close to the concentration range of 2-CHDA reported to be present in atherosclerotic lesions (10 \( \mu \)mol/L), a concentration that is almost 1400 times that of normal aorta samples. The plasmalogen content in LDL was found to be \( \approx 74 \) mmol/mg TC, respectively. HOCl treatment of LDL (oxidant:lipoprotein molar ratio of 400:1) also led to generation of 2-CHDA (data not shown). However, only up to 22% of LDL-associated plasmalogen was consumed after HOCl treatment.

Second, we tested whether HOCl—generated by the MPO-hydrogen peroxide-chloride system—will target the plasmalogen moiety of HDL, leading to the generation of 2-CHDA. The predominant in vivo sources for MPO released during the oxidative burst are neutrophils and monocytes, in which MPO comprises 5% and 1% in the total protein content, respectively. Here, we show that considerable amounts of 2-CHDA were generated via the active enzymatic pathway of stimulated neutrophils coincubated with native HDL (Figure 4A). In line with studies performed on monocytes, the presence of the MPO inhibitor sodium azide (Figure 4A) or the HOCl scavenger...
dichlorobenzimidazole 1-methyls showing eNOS mRNA stability in the presence of 5,6-blots. Analysis of eNOS expression (D) and Northern blot experiments (B) and confocal image of eNOS distribution (C). Western

Of importance, effects of 2-ClHDA on eNOS protein expression (Figure 4E), indicating that eNOS mRNA stability is impaired.

Increased eNOS mRNA expression in the presence of DRB (mean ± SE, n = 3) (E). For experimental details, see Materials and Methods.

methionine (data not shown) prevented 2-ClHDA formation after phorbol-ester activation of neutrophils.

To clarify whether 2-ClHDA directly alters eNOS expression and NO biosynthesis, 2-ClHDA was used in cell culture experiments. We present 4 lines of evidence that plasmalogen-derived 2-ClHDA is the lipid component mediating the inhibitory effects of HOCl-HDL on eNOS protein expression and activity. Normally, eNOS produces a tonic amount of NO, which is responsible for the homeostasis between the endothelium and surrounding tissues. Our data indicate that HOCl-mediated generation of chlorinated fatty aldehydes from lipoprotein-associated plasmalogens could represent a dominant pathway contributing to pathophysiological changes, ultimately leading to inflammatory responses, endothelial dysfunction, and tissue injury.

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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 g/ml) and HDL (d = 1.125 to 1.21 g/ml) was prepared by discontinuous density ultracentrifugation from plasma obtained from normolipidemic, healthy blood donors.\textsuperscript{8,26} HOCl-modification of lipoproteins (at indicated oxidant : lipoprotein molar ratios) and characterization of the modified lipoprotein particles was performed as described.\textsuperscript{8,13,26} HOCl-modified lipoproteins were stored at 4°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.\textsuperscript{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 µm 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.27 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 synthesized28 and purified as described29 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.30 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.
[14]C-L-Citrulline synthesis from cell lysates was estimated after preincubation of HUVECs with lipoproteins (18 h, 90 µg TC/ml) as described.31

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).24 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.24 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.