Hypochlorite-modified High Density Lipoprotein, a High Affinity Ligand to Scavenger Receptor Class B, Type I, Impairs High Density Lipoprotein-dependent Selective Lipid Uptake and Reverse Cholesterol Transport

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Hypochlorous acid/hypochlorite (HOCl/OCI⁻), a potent oxidant generated in vivo by the myeloperoxidase-H₂O₂-chloride system of activated phagocytes, alters the physiological properties of high density lipoprotein (HDL) by generating a proatherogenic lipoprotein particle. On endothelial cells lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) and scavenger receptor class B, type I (SR-BI), act in concert by mediating the holoparticle of and selective choleseryl ester uptake from HOCl-HDL. We therefore investigated the ligand specificity of HOCl-HDL to SR-BI-overexpressing Chinese hamster ovary cells. Binding of HOCl-HDL was saturable, and the degree of HOCl modification was the determining factor for increased binding affinity to SR-BI. Competition experiments further confirmed that HOCl-HDL binds with increased affinity to the same or overlapping domain(s) of SR-BI as does native HDL. Furthermore, SR-BI-mediated selective HDL-cholesteryl ester association as well as time- and concentration-dependent cholesterol efflux from SR-BI overexpressing Chinese hamster ovary cells, depending on the degree of HOCl modification of HDL, markedly impaired. The most significant findings of this study were that the presence of very low concentrations of HOCl-HDL severely impaired SR-BI-mediated bidirectional cholesterol flux mediated by native HDL. The colocalization of immunoreactive HOCl-modified epitopes with apolipoprotein A-I along with deposits of lipids in serial sections of human atheroma shown here indicates that the myeloperoxidase-H₂O₂-chloride system contributes to oxidative damage of HDL in vivo.

In a global sense, native high density lipoprotein (HDL) appears to function as a shuttle, transferring cholesterol between tissues and other lipoproteins (for review, see Refs. 1–3). Oxidation and chemical modification affect the ability of HDL to mediate reverse cholesterol transport and other events associated with the development of atherosclerosis (4, 5). One of the potential in vivo oxidants, generated only by the myeloperoxidase-H₂O₂-chloride system (6) of activated phagocytes, is hypochlorous acid/hypochlorite (HOCl/OCI⁻) (7). HOCl reacts with thiols and thioesters, Fe-S centers, nucleotides, with lipids, e.g. unsaturated fatty acids (FAs), to form chlorohydrins, protein-derived amine groups to form chloramines, and lipoproteins, respectively. HOCl-modified lipoproteins have several other atherogenic and/or proinflammatory features in vivo and in vitro, e.g. decrease of NO synthesis in endothelial cells (8), modulation of platelet reactivity (9, 10), and chemotactic migration of neutrophils (11). HOCl-modified lipoproteins induce leukocyte production of reactive oxygen species (12), cause endothelial leakage, and stimulate leukocyte adherence to, and migration into, the subendothelial space (13). Both HOCl-modified (lipoproteins and apolipoproteins, e.g. apolipoprotein B-100, have been identified in human and rabbit lesion material (14–17), and in situ staining has revealed colocalization of myeloperoxidase and HOCl-modified epitopes with endothelial cells and monocytes/macrophages (15, 18, 19). The fact that scavenging of myeloperoxidase-generated HOCl significantly impairs lesion severity (20) supports the notion that both HOCl as well as HOCl-modified (lipoproteins account for deleterious effects during inflammatory conditions (21–26). In vitro experiments further demonstrated that HOCl transforms native HDL into a “high uptake” form for macrophages (27). Compared with native HDL, internalization and degradation of HOCl-HDL by macrophages was enhanced, whereas lipoprotein-mediated cholesterol efflux, regardless of whether reagent NaOCl or myeloperoxidase-generated HOCl was used to modify the lipoprotein, was decreased (27, 28). Recently, lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) (29) and scavenger receptor class B, type I (SR-BI; for review, see Ref. 30), were identified on human umbilical endothelial cells as candidate receptors that mediate holoparticle of and selective cholesteryl ester (CE) uptake from HOCl-HDL in concert (31).

Therefore, the present study was aimed at investigating the binding properties of HOCl-HDL to SR-BI in more detail. During these in vitro experiments with SR-BI-overexpressing Chinese hamster ovary cells lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) and scavenger receptor class B, type I (SR-BI; for review, see Ref. 30), were identified on native and HOCl-modified HDL, internalization and degradation of HOCl-HDL by macrophages was enhanced, whereas lipoprotein-mediated cholesterol efflux, regardless of whether reagent NaOCl or myeloperoxidase-generated HOCl was used to modify the lipoprotein, was decreased (27, 28). Consequently, lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) and scavenger receptor class B, type I (SR-BI; for review, see Ref. 30), were identified on human umbilical endothelial cells as candidate receptors that mediate holoparticle of and selective cholesteryl ester (CE) uptake from HOCl-HDL in concert (31).

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nese hamster ovary (CHO) cells (32) we were able to demonstrate that (i) HOCl-HDL is a high affinity ligand for SR-BI; (ii) the degree of HOCl modification controls the efficiency of CE uptake from and cellular cholesterol efflux to the modified lipoprotein particles. From our in vitro findings we conclude that modification of HDL by HOCl may block SR-BI-mediated interaction with native HDL.

**EXPERIMENTAL PROCEDURES**

**Materials**

NaOCl, organic solvents, potassium bromide, and egg yolk lecithin were obtained from Sigma. Radiochemicals were purchased from PerkinElmer Life Sciences. DMEM and Ham’s F12K medium were from Invitrogen, and and fetal calf serum was obtained from Roche Molecular Biochemicals. Plasticware used for tissue culture was obtained from Costar. Triethyamine was purchased from Fluka. Acetone and methanol were of HPLC grade (Lactan, Graz, Austria). All other chemicals were obtained from Merck except where indicated.

**Methods**

**Human Lipoproteins**

HDL (subclass 3, d = 1.215–1.217 g/ml) was prepared by discontinuous density ultracentrifugation of plasma obtained from normaligid donors (33). HDL was recovered from the tubes and diaanalyzed against phosphate-buffered saline (PBS; 10 mM, pH 7.4, 0.15 M NaCl). The protein concentration of the resulting solution was determined by the Lowry procedure (34).

**NaOCl Modification of Lipoproteins**

Modification of HDL with OCl⁻ was performed as described by Panzenbock et al. (27). Briefly, 1 mg of HDL-protein/ml of PBS was incubated with NaOCl solution (a single addition and gentle vortexing) at 37°C for 60 min, 0°C, under argon) with the final pH adjusted to 7.4. The modified HDL preparations were passed over a PD10 column to remove unreacted NaOCl. The concentration of reagent NaOCl was determined spectrophotometrically using a molar absorption coefficient for OCl⁻ of 350 cm⁻¹ at 292 nm (28). The modified HDL preparations were stored at 4°C and used between 6 and 24 h after exposure to the oxidant.

**Amino Acid Analysis**

Aliquots of native and NaOCl-modified HDL (450 μg of protein) were lyophilized in 5-ml ampoules and purged with nitrogen before hydrolysis in constant boiling 6 N HCl (24 h, 120°C). Amino acid analysis was performed on a Biotronics analyzer as described by Panzenbock et al. (27).

**HPLC Analysis of Phospholipids**

Phospholipid (PL) analysis was performed on a Hewlett-Packard 1050 system equipped with a Varex light-scattering detector ELSD IIA with a Vertex column (LiChrospher Diol 100, 5 μm, 250 × 2.0 mm, Knauer). The atomizer inlet air flow (60 mm) and drift tube temperature (120°C) of the detector were maintained throughout all analyses. Separations were performed by gradient elution (flow rate, 0.8 ml/min) using solvent systems of n-hexane, methanol:acetic acid:triethyamine (765:7:15:31:12.9, v/v), and acetone:acetic acid:triethyamine (765:4.15:31:12.9, v/v) as described by Sas et al. (35). Eluents were flushed continuously with helium.

**LPLs (phosphatidylcholine (PC), phosphatidylinositol, phosphatidic acid, phosphatidylearaine, and phosphatidylethanolamine) and the corresponding lysosphospholipids were used for calibration curves were extracted from wild type yeast cells grown on full medium and separated by two-dimensional thin-layer chromatography on silica gel 60 plates by using chloroform, methanol, and 25% ammonia (65:35:5, v/v/v) as first and chloroform:methanol:acetic acid:water (50:20:10:10: 5, v/v/v) as second developing solvents. Determination of PL content was performed by a standard phosphorus quantification procedure as described by Broekhuysen (36).

**Fatty Acid and Cholesterol Analysis**

FA analysis of total HDL lipids was performed as described by Sattler et al. (37, 38). The FA composition of HDL lipid subclasses was analyzed after in situ transesterification of the corresponding TLC spots. Separation of FA methyl esters was performed on a WCOT fused silica 25 m FFAP-CB column (0.32-mm inner diameter, Chrompack) using a HP 5890 gas chromatograph equipped with a flame ionization detector and a split/splitless injector (Hewlett-Packard). Cholesterol analysis (esterified and free cholesterol) of HDL lipids was performed as described by Panzenbock et al. (27).

**Lipoprotein Labeling Procedures**

**HDL Labeling with Na²¹²⁵I—**Iodination of HDL and HOCl-HDL was performed as described by Artl et al. (39) using NaBr-succinimide (40) as the coupling agent. Routinely, 1 μCi of Na²¹²⁵I was used to label 5 mg of HDL. This procedure resulted in specific activities between 300 and 500 dpm/μg of protein with less than 3% lipid-associated activity. No cross-linking or fragmentation of apolipoprotein A-I (apoA-I, the major apolipoprotein of HDL) resulting from the iodination procedure could be detected by SDS-PAGE and subsequent autoradiography. HDL Labeling with [³H]cholesterol—HDL and HOCl-HDL were labeled with [cholesterol]-1,2,6,7-³H]palmitate by CE transfer protein-catalyzed transfer from donor liposomes as described by Artl et al. (39). Briefly, 200 μCi of the corresponding label and 100 μg of egg yolk lecithin were dried under argon, followed by the addition of 1 ml of PBS. The mixture was shaken for 2 min at 37°C and sonicated. Lipoproteins (1 ml, containing 3–6 mg of protein), 1 ml of lipid-deficient serum (as a source of CE transfer protein) 1 ml of PBS were added. The mixture was incubated under argon at 37°C in a shaking water bath overnight. Subsequently, the labeled HDL fractions were reisolated in a TLX120 bench top ultracentrifuge in a TL100.4 rotor (Beckman). The HDL band (d = 1.215–1.217 g/ml) was aspirated and dialyzed against 10 mM PBS, pH 7.4. This labeling procedure resulted in specific activities of 5–9 cpm/μg of protein.

**Cell Culture Studies**

**CHO Cells—**Ida cells (clone 7, a low density lipoprotein receptor-deficient CHO cell line) were cultured in Ham’s F12K medium containing 5% (v/v) fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (32, 41). Stable transfectants expressing murine SR-BI (Ida[mSR-BI]) were maintained in medium containing 0.5 mg/ml G418. Ida[mSR-BI] showed an approximately 50-fold higher expression of SR-BI protein (estimated by immunoblotting experiments, data not shown) compared with Ida[Δ]. Both cell lines were kindly provided by Dr. Monty Krieger, MIT.

Association and Degradation Studies—Association studies of HDL and HOCl-HDL to CHO cells were performed at 37°C with increasing amounts of [³H]CE-labeled lipoproteins in the absence (total association) or presence of 1 mg of protein/ml (unspecific association) of unlabeled analogous lipoprotein (42). Labeled lipoproteins added to a final concentration up to 500 μg of protein/ml, and cells were incubated in DMEM without fetal calf serum at 37°C up to 2 h. After this incubation the medium was aspirated, and the cells were rinsed twice with ³H-labeled saline (containing 5% (w/v) bovine serum albumin) by following two washes with ³H-labeled saline. Cells were lysed with 0.3 N NaOH. The radioactivity and protein content of the cell lysate were measured in the same aliquot. Protein was measured according to Lowry et al. (34). Specific association was calculated as the difference between the total and nonspecific association (42).

Degradation of [³H]labeled HDL particles by CHO cells was estimated by measuring the non-trichloroacetic acid-p precipitable radioactivity in the medium after precipitation of free iodine with AgNO₃ (43). Briefly, 0.5 ml of medium was removed, mixed with 100 μl of bovine serum albumin (30 mg/ml), and 1 ml of trichloroacetic acid (3, 4°C) and left at 4°C for 30 min. Subsequently, 200 μl of an AgNO₃ solution (0.7 M) was added and mixed. Then the samples were centrifuged at 3,000 rpm at 4°C for 15 min. The radioactivity of the supernatant was determined using a gamma-counter (125I). Specific degradation was calculated as the difference between the total and non-specific degradation (39).

Efflux Experiments—Efflux of [³H]labeled cholesterol from CHO cells was measured by appearance of [³H]cholesterol in the cellular supernatant. The cells were incubated in the presence of 1 μCi/ml of [³H]cholesterol for 48 h. After cholesterol loading of the cells, the monolayer were rinsed twice with ³H-labeled saline (containing 5% (w/v) bovine serum albumin) and twice with ³H-labeled saline (39). Efflux experiments were initiated by the addition of HDL, HOCl-HDL, or apoA-I in DMEM without fetal calf serum. At the indicated time points (up to 4 h) the medium was collected and counted on a β-counter. Afterwards, the
cells were lysed in 0.3 n NaOH to estimate the cellular protein content and the cell-associated radioactivity. Efflux of radioactive label into the medium was calculated as the percentage of radioactivity associated with cells before the addition of the indicated cholesterol acceptors.

Both HDL-mediated cellular cholesterol efflux and CE association by ldlA[mSR-BI] cells were studied in the presence of increasing amounts of KKB-1, a polyclonal rabbit anti-mSR-BI antiserum, reported to block both CE association and cellular cholesterol efflux (43).

Sources of Human Tissue

Aortas and aorta abdominals of three autopsy cases with different histological classification of atherosclerosis (lesion types III and IV according to Ref. 45) who died of cerebral hemorrhage were used. The morphology of the aortas investigated ranged from microscopically normal morphology to thickened intima up to pronounced atheroma containing calcium inclusion. The autopsy material was taken 12 h after death.

Immunohistochemistry was carried out on tissues embedded in tissue freezing medium (Tissue Tec OCT compound, Miledi, Elkhart, IN). Serial cryosections (5 μm, Microm, Walldorf, Germany, Microm HM 500 OM) were collected on glass slides, air dried for 2 h at 25 °C, fixed in acetone for 5 min at 25 °C, and stored at −70 °C until required.

Lipids were stained with Oil Red O. After staining the nuclei with hemalum, sections were mounted with Kaiser’s glyceroen gelatin. Pictures were taken on an Axiophot microscope (Zeiss, Oberkochen, Germany) by using the digital camera AxiCam HRc (Zeiss).

Double Immunofluorescence and Confocal Laser Scanning Microscopy

For double immunofluorescence the sections were thawed, fixed once more in acetone at 25 °C (5 min), and rehydrated in PBS (25). After blocking (10 min with human AB serum, diluted 1:10 in blocking solution, Dako, Vienna), the sections were incubated with monoclonal antibody 2D10659 (dilution 1:10, specifically recognizing HOCl-modified epitopes; Ref. 46) for 30 min at 25 °C, followed by an incubation step with biotinylated goat anti-mouse IgG (LSAB kit, Dako) for 15 min and cyanine (Cy)-3 labeled streptavidin (Amersham Biosciences, dilution 1:300). Afterwards the sections were blocked with rabbit nonimmune serum (dilution 1:300, Dako) and incubated (30 min) with rabbit anti-human apoA-I antiserum (dilution 1:1,000, Behring) followed by an incubation with Cy-2-labeled goat anti-rabbit IgG (dilution 1:300, Jackson Dianova). PBS was used for washing sections between different incubation steps. Sections were mounted with Moviol (Calbiochem-Novabiochem) and analyzed on a confocal laser scanning microscope (Leica TCS NT, Leica Lasertechnik GmbH, Heidelberg) equipped with an argon-krypton laser (25). Signals were detected with a double dichroic beam splitter (488/568 nm), using a 580 nm short pass and a 530 nm band pass filter for Cy-2 and a 590 nm long pass filter for Cy-3. Control experiments encompassed immunofluorescence (i) without primary detection antibodies, (ii) with monoclonal or polyclonal nonimmune antibodies as primary antibodies, and (iii) without secondary antibodies.

RESULTS

Characterization of the Protein and Lipid Moiety of HOCl-HDL—SDS-PAGE and subsequent silver staining of apolipoproteins revealed that an increasing oxidant:HDL molar ratio resulted in a gradual loss of monomeric, 28-kDa apoA-I (Fig. 1), with the concomitant formation of high molecular mass aggregates even at low HOCl concentrations (25:1). To identify preferential amino acid targets, the amino acid composition of HOCl-HDL was analyzed. From the data shown in Table I, it is evident that Cys and Met were completely consumed at a molar HOCl:HDL ratio of 50:1, whereas His, Arg, and Lys were consumed in a dose-dependent fashion up to 20–50% at the highest HOCl concentration used (Table I). As a consequence, the relative electrophoretic mobility (46) of the modified lipoprotein particles in agarose gel electrophoresis increased.

Characterization of the lipid moiety of native HDL revealed that PC is the major PL species accounting up to 90% of total PLs, whereas the remaining PL species are composed of phosphatidylinositol, phosphatidic acid, phosphatidylethanolamine, and phosphatidylethanolamine, respectively. Modification of HDL by increasing the HOCl concentrations led to a slight decrease of PC (Table I), whereas in parallel only trace amounts of lyso-PC were formed (up to 1.2% of total PC at the highest HOCl concentration used, 200:1). The amount of the minor PL species was not altered significantly during the HOCl treatment (data not shown). In line with our previous findings (27) the amount of unsaturated FAs (primarily C18:1 and C18:2) in HDL subclasses (PL and CE fraction) was decreased slightly (approximately 10%) at the highest HOCl concentration (200:1) used. In parallel, the amount of free FAs remained widely unaltered at the highest HOCl concentration used (data not shown). Further characterization of the lipid moiety revealed that an increasing NaOCl concentration was paralleled by a marginal increase of the unesterified cholesterol content. Under the experimental conditions described in Table I and Fig. 1, no formation of lipid peroxidation products (measured as thio-barbituric acid-reactive substances) could be detected.

Cell Association Studies—To investigate binding properties of lipoproteins to ldlA[mSR-BI] and ldlA7 cells at 37 °C, the protein moiety of the lipoproteins was labeled with Na<sup>125</sup>I. Binding capacity (B<sub>max</sub> values) of <sup>125</sup>I-HDL and <sup>125</sup>I-HOCl-HDL to ldlA[mSR-BI] cells was about 12–40-fold higher compared with ldlA7 cells (data not shown), indicating that SR-BI mediates binding of HDL and HOCl-HDL, respectively (Fig. 2A). Nonlinear regression analysis showed a marked increase in binding affinity (K<sub>d</sub> values, Table II) for HOCl-HDL with increasing degree of HOCl modification but decreased B<sub>max</sub> values. Time-dependent association studies (Fig. 2B) revealed that <sup>125</sup>I-HOCl-HDL associates much faster with SR-BI compared with native HDL (Table II).

Competition Experiments—This series of experiments was performed to obtain information about the binding specificity of the recognition site(s) for HOCl-HDL to SR-BI. HOCl-HDL (at a different oxidant:HDL molar ratio) inhibited <sup>125</sup>I-HDL cell association to SR-BI (Fig. 3) in a concentration-dependent manner; the calculated IC<sub>50</sub> values were 20 μg/ml for native HDL and 5 μg/ml (oxidant:lipoprotein molar ratio of 50:1) and 1.2 μg/ml (oxidant:lipoprotein molar ratio of 200:1) for HOCl-HDL. From these experiments it is evident that the degree of HOCl modification is a determining factor that governs the displacement capability for native HDL from SR-BI. Both binding studies (Fig. 2) and competition experiments (Fig. 3) revealed that <sup>125</sup>I-labeled HOCl-HDL binds with increased affinity to the same or overlapping domain(s) of SR-BI as does native HDL.

Comparison of HDL-CE Association as a Function of HOCl Modification—To compare the efficiency of CE uptake, HDL and HOCl-HDL were double labeled in the lipid and protein moiety and the cell association of the tracer was studied at conditions where binding of lipoproteins (100 μg of protein/ml) to SR-BI is saturated (Fig. 4A). For HDL and HOCl-HDL, <sup>3</sup>H]CE uptake (expressed as apparent particle uptake) ex-
Characterization of native and HOCI-modified HDL

Amino acid analysis was performed as under "Methods." Values obtained with ldlA7 cells were subtracted from the ranges of the measurements.

| Lipoprotein (μg protein/ml) | HDL | HOCl-HDL (50) | HOCl-HDL (200) |
|----------------------------|-----|---------------|----------------|
| HDL                        | 1.1 | 4.5           | 23.6           |
| HOCl-HDL (mol:mol)         | 25:1| 0.5           | 2.5            |
| 50:1                       | 0   | 0             | 19.3           |
| 100:1                      | 0   | 0             | 18.8           |
| 200:1                      | 0   | 0             | 11.1           |

Results are expressed in mol of amino acids/mol of HDL. TC (total cholesterol, 100% = 305 μg of TC/mg of HDL protein) and unesterified cholesterol (FC, 100% = 33 μg of FC/mg of HDL protein) was determined as described under "Methods." FC (100% = 352 μg of FC/mg of HDL protein) content of HDL and HOCl-HDL was determined by HPLC analysis as described under "Methods." REM, relative electrophoretic mobility.

Calculation of $K_d$, $B_{max}$, and $t_{1/2}$ values by nonlinear regression analysis

| Lipoprotein (μg protein/ml) | $K_d$ (μg/ml) | $B_{max}$ (ng/mg) | $t_{1/2}$ (min) |
|----------------------------|---------------|-------------------|-----------------|
| HDL                        | 15.2 ± 1.4    | 596 ± 25          | 31 ± 7          |
| HOCl-HDL                   | 50:1          | 5.7 ± 0.3         | 459 ± 12        | 9.3 ± 0.7       |
|                            | 200:1         | 1.2 ± 0.2         | 312 ± 20        | 2.9 ± 0.3       |

* Time necessary to reach half-maximal cell association.

FIG. 2. Specific cell association of [125I]-labeled HOCl-modified lipoproteins to SR-BI. A, ldl[msr-BI] and ldlA7 cells were incubated in DMEM for 2 h at 37 °C with increasing concentrations of [125I]-HDL and [125I]-HOCl-HDL (0.2–25 μg of protein/ml). B, Ldl[msr-BI] and ldlA7 cells were incubated in DMEM for the indicated time points in the presence of 10 μg/ml [125I]-HDL and [125I]-HOCl-HDL. The oxidant:lipoprotein molar ratio of HOCl-HDL was 50:1 and 200:1. After 2 h the cells were washed and lysed in 0.3 N NaOH to determine the amount of associated label and the cellular protein content. Specific association was determined as described under "Methods." Only specific data are shown. Values obtained with ldlA7 cells were subtracted from ldl[msr-BI] cells. Results are the average of triplicate determinations from one representative experiment of three, and error bars represent the ranges of the measurements.

HOCl-HDL uptake

HOCl-HDL was used to determine the association of HDL and HOCl-HDL to SR-BI. It is evident that at all time points uptake as described for native HDL (30). When analyzed in terms of CE uptake/ng of protein bound within 2 h, native HDL shows the most efficient selective CE uptake (1.25 ng of CE/ng of protein), whereas HOCl-HDL is less efficient (1.12 and 0.95 ng of CE/ng of protein, a molar excess of oxidant was 50:1 and 200:1). Fig. 4B shows that the capacity for CE uptake from HOCl-HDL and native HDL up to 20 μg/ml was similar, although the cell association of HOCl-HDL to SR-BI is increased at low lipoprotein concentrations (Fig. 2A). At higher lipoprotein concentrations CE uptake from HOCl-HDL was, depending upon the degree of HOCl modification, remarkably impaired compared with native HDL seemingly because the $B_{max}$ values (Table II) of HOCl-HDL are impaired, and therefore one could expect that the reduction of selective HOCl-HDL CE uptake at higher lipoprotein concentrations would be more pronounced.

Cholesterol Efflux Experiments—The next series of experiments was aimed at studying the effects of HOCl modification on the ability of these lipoprotein particles to promote SR-BI-mediated cholesterol efflux. The capacity of these lipoprotein particles to promote SR-BI-mediated cholesterol efflux is evident that at all time points

TABLE I

Characterization of native and HOCl-modified HDL

Table showing the characterization of native and HOCl-modified HDL.

| Cys | Met | Tyr | Lys | His | Arg | REM | PC | TC | FC |
|-----|-----|-----|-----|-----|-----|-----|----|----|----|
| HDL | 1.1 | 4.5 | 23.6| 43.4| 7.0 | 27.1| 1  | 100| 100| 100|
| HOCl-HDL (mol:mol) | 25:1 | 0.5 | 2.5 | 21.1| 41.6| 7.0 | 26.6| 1.05| 98.8 ± 1 | 99.4 ± 2 | 100 ± 2 |
|     | 50:1 | 0   | 0   | 19.3| 38.0| 6.9 | 26.6| 1.23| 95.8 ± 2 | 99.3 ± 1.7 | 101 ± 1.7 |
|     | 100:1| 0   | 0   | 18.8| 29.3| 6.4 | 26.7| 1.57| 95.0 ± 1 | 94.8 ± 2.5 | 104 ± 2.5 |
|     | 200:1| 0   | 0   | 11.1| 25.8| 5.1 | 23.7| 1.79| 92.4 ± 2 | 90.7 ± 3 | 107 ± 3 |

Methods.

FIG. 3. Competition studies. Ldl[msr-BI] cells were incubated in DMEM for 2 h at 37 °C with 10 μg of protein/ml of [125I]-HDL in the presence of increasing concentrations of unlabeled competitors (HDL and HOCl-HDL (oxidant:lipoprotein molar ratio of 50:1 and 200:1)). Values are the averages of triplicate determinations from one representative experiment of three, and error bars represent the ranges of the measurements.
Inhibition of Selective CE Uptake from and Cholesterol Efflux to Native HDL by HOCl-HDL—Gu and co-workers (44) have shown that SR-BI-mediated cholesterol efflux is dependent on lipoprotein binding to the receptor. These authors (44) demonstrated that blocking of SR-BI by a specific antibody inhibited binding of and lipid uptake from HDL. In parallel, the cellular cholesterol efflux to HDL was strongly inhibited. We therefore examined to what extent HOCl-modified HDL is able to block SR-BI-mediated functions that are normally brought about by native HDL. At different oxidant:lipoprotein molar ratios (50-200-fold molar HOCl excess) HOCl-HDL inhibited HDL-mediated efflux by 22 and 53% (depending upon the oxidant:lipoprotein molar ratio), respectively. The ability of HOCl-HDL to block the interaction of SR-BI with HDL can be mimicked by a specific antiserum raised against SR-BI. Addition of this antiserum inhibited up
to 80% of HDL-mediated CE uptake (Fig. 6C) and approximately 60% of the cellular [3H]cholesterol efflux (Fig. 6D). Because cellular cholesterol efflux cannot be inhibited completely either by HOCl-HDL or the specific antiserum it is reasonable to assume that part of cellular cholesterol efflux to HDL can occur independently of lipoprotein binding to SR-BI (49).

**Immunohistochemistry**—In nondiseased vessels, faint but detectable colocalization of HOCl-modified epitopes with apoA-I was found in the endothelial lining of areas with normal morphology (Fig. 7A). In line with our previous findings (15), atheromas always showed pronounced staining for HOCl-modified epitopes (red) and apoA-I (green) on the endothelial layer of a morphologically normal section (scanned field areas (x/y dimension): 500 μm). B–D, atheromas (type IV lesion) (B) pronounced staining for HOCl-modified epitopes (red) and apoA-I (green) could be observed (scanned field areas (x/y dimension): 500 μm). C, enlargement of B clearly demonstrates pronounced staining of HOCl-modified epitopes or apoA-I in the connective tissue. Colocalization of HOCl-modified epitopes with apoA-I was primarily present in lipid-rich areas (see D; scanned field areas (x/y dimension): 100 μm). D, serial section of C stained with Oil red O (original magnification: ×100 μm).

Fig. 6. Blocking of SR-BI-mediated interaction with native HDL. LDL[mSR-BI] cells were incubated with 100 μg/ml [3H]CE-labeled HDL for 2 h at 37°C in the presence of increasing concentrations of unlabeled competitors (HDL and HOCl-HDL) (oxidant:lipoprotein molar ratio of 50:1 and 200:1) (A) or increasing concentrations of a polyclonal anti-mSR-BI antiserum or nonimmune serum (C). Values are the averages of triplicate determinations from one representative experiment of two, and error bars represent the ranges of the measurements. 100% = 700 ng of HDL-CE/mg of cell protein. B. LDL[mSR-BI] cells were labeled with [3H]cholesterol as described under "Methods" and incubated with 100 μg of protein/ml of HDL for 2 h in the presence of increasing concentrations of indicated competitors (HDL and HOCl-HDL (oxidant:lipoprotein molar ratio of 50:1 and 200:1)) or increasing concentrations of a polyclonal anti-mSR-BI antiserum or nonimmune serum (D). Values are the averages of triplicate determinations from one representative experiment of four, and error bars represent the ranges of the measurements.
HOCI-modified epitopes and apoA-I were always associated with lipid deposits containing either lipid droplets (Fig. 7D) or cholesterol crystals.

**DISCUSSION**

We have recently reported selective CE uptake from HOCI-HDL via an SR-BI-mediated pathway in human umbilical venous endothelial cells (31). These findings prompted us to study the interaction of HOCI-HDL with this receptor in more detail. Association studies performed with $^{35}$S-labeled lipoprotein particles revealed that binding affinity of HOCI-HDL to SR-BI was dependent on the degree of HOCI modification. Competition studies clearly showed that the binding domain(s) of HOCI-HDL overlap or are identical with binding domains for native HDL. Furthermore, we could demonstrate that the efficiency for SR-BI-mediated lipid tracer uptake is inversely related to the degree of HOCI modification.

Previous findings suggested that apoA-I conformation (which is dependent on the particle diameter) markedly influences HDL interaction with SR-BI (50). de Beer et al. (51) demonstrated that binding affinity of reconstituted HDL (containing the same amounts of apoA-I as did native HDL) was significantly greater for the larger (9.6 nm) particle than for the 7.8-nm particle. In parallel, the negative charge of the corresponding 9.6-nm particle was increased. In line with these studies, the modification of HDL by HOCl leads to conformational changes in apo-A-I. Besides formation of cross-linked apoA-I (see Fig. 1 and Ref. 27) and formation of A-I/A-II homo- and heterodimers, the increased negative surface charge (Table II) of HOCl-modified HDL particles and the slight percentage increase in particle diameter (HOCI-HDL versus HDL) (27) are comparable with those reported for large reconstituted HDL particles. In line with previous studies (51), the binding affinity of HOCl-HDL was also increased up to 15-fold compared with native HDL (Table II).

Oxidation/modification of HDL was demonstrated to result almost exclusively in diminished cholesterol efflux capacity because of changes in the protein and the lipid moiety. Only oxidative tyrosylation appears to be an exception because tyrosyl radical modification of HDL enhanced cholesterol mobilization and efflux (5). We (28) and others (52) could show that the amount of PLs (a determining factor for binding of the lipoprotein particle to SR-BI) was not altered markedly during treatment of HDL with HOCI (Table II). In addition to the lipid moiety, the presence of amphipathic helices with high lipid binding affinity determine the ability of apolipoproteins to participate in the membrane microsolubilization of cellular PLs and cholesterol (53). Therefore it was suggested that the functional integrity of apoA-I and specific amino acid stretches (54–57) participate in HDL-mediated cholesterol efflux. All of these epitopes that are involved in HDL-mediated cellular cholesterol efflux (54–57) contain amino acids that are highly susceptible to HOCl oxidation/modification (27, 58). Liu and co-workers (59) recently demonstrated that mutations in helices 4 and 6 of apoA-I can result in high affinity binding of reconstituted particles to SR-BI; however, these particles do not support efficient cellular cholesterol efflux. These authors (59) suggested that a productive complex between reconstituted particles and SR-BI is required for efficient lipid transport; mutation in apoA-I could lead to “nonproductive” binding to SR-BI. It is noteworthy mentioning that helices 4 and 6 contain methionine (and lysine) residues, which are highly susceptible to HOCI treatment. One of our previous studies (58) as well as amino acid analysis performed here (Table I) and matrix-assisted laser desorption ionization time-of-flight analysis revealed that methionine residues present in HDL-associated apoA-I and apoA-II, respectively, are fully oxidized to corresponding methionine sulfoxides even at low (25:1–50:1) HOCI concentrations. Therefore, we suggest that HOCI modification of HDL could also lead to a nonproductive binding of HDL to SR-BI which does not support efficient bidirectional lipid transfer.

Probably the most remarkable observation obtained during the present study is the low concentration of HOCI-HDL necessary to inhibit cellular cholesterol mobilization by native HDL. Our results revealed that only 10 μg of protein/ml of HOCI-HDL inhibited cholesterol efflux (initiated by 100 μg of HDL/ml) by more than 50%. This indicates that HOCI modification of only a small portion of HDL could significantly interfere with SR-BI-dependent reverse cholesterol transport where high HOCI concentrations are generated (60). Based on in vitro experiments (7, 61) the HOCI concentration at sites of acute inflammation were calculated to be in the range of 340 μM or above (60). Assuming HDL$_3$ plasma concentrations of ~6–12 μM (and it is conceivable that subendothelial concentrations would be lower) one would yield a minimum estimate of HOCI: HDL$_3$ ratios of ~30:1–50:1.

From our findings we propose that HOCI-HDL is able to compete effectively for physiologically relevant functions of SR-BI. Our findings are supported by previous observations (44, 59) that SR-BI-mediated $[^3H]$cholesterol efflux to HDL depends critically on ligand binding to this receptor, which indicates that the mechanisms of selective lipid uptake and cholesterol efflux may be intimately related. Expression of SR-BI in atherosclerotic plaque (62) has been considered antiatherogenic because of HDL-mediated reverse cholesterol transport from peripheral tissues. The fact that HOCI-modified epitopes are present in human lesion material (14, 15, Fig. 7) could favor impaired (SR-BI-mediated) cholesterol efflux from macrophages. Subsequently, HOCI-modified epitopes are abundantly present in the liver (63), and impairment of SR-BI-mediated CE uptake by this organ could lead to a decreased clearance of native/modified CEs (64–66). The fact that both tissue macrophages and Kupffer cells, both known to express SR-BI (62, 67), express myeloperoxidase (18, 19, 63) underscores the potential of these cells to generate HOCI under in vivo conditions. In situ colocalization of HOCI-modified epitopes with apoA-I (Fig. 7) supports the concept that HDL and apoA-I, abundantly present in lesion material, is prone to be modified by HOCI under in vivo conditions.

Summarizing our findings, we propose that modification of HDL by HOCI significantly alters the physiological properties of native HDL by two different mechanisms. First, direct modification of HDL by HOCI might impair the antiatherogenic properties of HDL during reverse cholesterol transport. Second, once modified the HOCI-HDL particle may exert indirect effects by acting as a highly potent competitor for the physiological duties performed by the interaction of native HDL with SR-BI.

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