Class B Scavenger Receptors CD36 and SR-BI Are Receptors for Hypochlorite-modified Low Density Lipoprotein*

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The presence of HOCl-modified epitopes inside and outside monocytes/macrophages and the presence of HOCl-modified apolipoprotein B in atherosclerotic lesions has initiated the present study to identify scavenger receptors that bind and internalize HOCl-low density lipoprotein (LDL). The uptake of HOCl-LDL by THP-1 macrophages was not saturable and led to cholesterol/cholesterol ester accumulation. HOCl-LDL is not aggregated in culture medium, as measured by dynamic light scattering experiments, but internalization of HOCl-LDL could be inhibited in part by cytochalasin D, a microfilament disrupting agent. This indicates that HOCl-LDL is partially internalized by a pathway resembling phagocytosis-like internalization (in part by fluid-phase endocytosis) as measured with [14C]glucose uptake. In contrast to uptake studies, binding of HOCl-LDL to THP-1 cells at 4 °C was specific and saturable, indicating that binding proteins and/or receptors are involved. Competition studies on THP-1 macrophages showed that HOCl-LDL does not compete for the uptake of acetylated LDL (a ligand to scavenger receptor class A) but strongly inhibits the uptake of copper-oxidized LDL (a ligand to CD36 and SR-BI). The binding specificity of HOCl-LDL to class B scavenger receptors could be demonstrated by Chinese hamster ovary cells overexpressing CD36 and SR-BI and specific blocking antibodies. The lipid moiety isolated from the HOCl-LDL particle did not compete for cell association of labeled HOCl-LDL to CD36 or SR-BI, suggesting that the protein moiety of HOCl-LDL is responsible for receptor recognition. Experiments with Chinese hamster ovary cells overexpressing scavenger receptor class A, type I, confirmed that LDL modified at physiologically relevant HOCl concentrations is not recognized by this receptor.

The oxidation of low density lipoprotein (LDL) has been proposed as a biological process that initiates and accelerates arterial lesion development (for review, see Refs. 1 and 2). Oxidized LDL (ox-LDL) accumulates in lesions and is present at other inflammatory sites. Recent findings revealed that monocyte-derived macrophages are likely candidates to mediate the in vivo oxidation of LDL. There are multiple potential pathways through which monocytes/macrophages may promote extracellular oxidation of LDL, but in principal non-enzymatic and enzymatic cellular mechanisms of LDL oxidation do exist. Although formation of the superoxide anion radical and/or its dismutation product, hydrogen peroxide, by monocyte-derived macrophages appears to be essential for LDL oxidation, a cell-derived factor that does participate in phagocyte-dependent oxidation of LDL is myeloperoxidase (MPO).

MPO is an abundant heme protein (for review, see Refs. 3 and 4) released during the oxidative burst by activated neutrophils and monocytes. A major function of MPO is to hold a central role in microbial killing, and recent findings revealed an association between MPO levels and the risk of coronary artery disease (5). MPO is also present in tissue macrophages such as those in vascular lesions (6–8). The MPO-hydrogen peroxide system plays a specific role in monocyte/macrophage-mediated oxidation of LDL by three major pathways. First, MPO catalyzes oxidation of l-tyrosine, generating the tyrosyl radical that may initiate dityrosine cross-linking of proteins or initiate LDL lipid peroxidation (for review, see Ref. 9). Second, MPO may use nitrite, the major end product of nitric oxide radical metabolism, as a substrate to nitrate (lipo)protein tyrosine residues and to initiate lipid peroxidation (10, 11). MPO may also generate a nitrating intermediate through secondary reaction of hypochlorous acid/hypochlorite (HOCl/OCl−) with nitrite, presumably forming nitryl chloride as a reactive intermediate (10). Third, because of its high concentrations in biological matrices, chloride is the preferred substrate for MPO, and HOCl/OCl−, a potent chlorinating oxidant, is formed. Under acidic conditions chloride gas is formed, leading to generation of chlorotyrosine (12). Alternatively, MPO-generated HOCl oxidizes free α-amino acids to aldehydes (13), leading to advanced glycation products present in human lesion material (14). However, the most common reaction of HOCl is with free amino groups of (apolipo)proteins, leading to formation of chloramines.

LDL modified by HOCl (HOCl-LDL) displays a number of apolipoprotein; CE, cholesterol ester; CHO, Chinese hamster ovary; FCS, fetal calf serum; hCD36, human CD36; HOCl/OCl−, hypochlorous acid/hypochlorite; MPO, myeloperoxidase, mSR-AI, murine scavenger receptor class A, type I; mSR-BI, murine scavenger receptor class B, type I; TBS, Tris-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline.
pathophysiological effects on phagocytes and vascular cells, contributing to the initiation and maintenance of the inflammatory process during the early phase of atherosclerotic lesion development. HOCl-LDL induces chemokine release of monocytes and chemotactic migration of neutrophils (15), initiates the respiratory burst of macrophages (16), stimulates polymorphonuclear leukocytes to an enhanced production of superoxide anion radical and hydrogen peroxide, enhances neutrophil degranulation (17), and inactivates lysosomal proteases (18). HOCl-LDL further decreases nitric oxide-synthesis in endothelial cells (19), causes endothelial leakage and stimulates leukocyte adherence to, and migration into, the subendothelial space (20). HOCl-LDL enhances platelet reactivity and release reaction (21, 22), and most importantly, HOCl converts LDL into a high uptake form for mouse peritoneal macrophages (23), leading to the formation of cholesteryl ester (CE)-laden foam cells, which are the hallmark of fatty streaks and the earliest recognizable lesion of atherosclerosis. The presence of 3-chlorotyrosine in human atherosclerotic lesions (12), the presence of HOCl-modified epitopes inside and outside macrophages/macrophages, endothelial cells, and smooth muscle cells in human and rabbit lesion material (7, 24, 25), and the presence of HOCl-modified apolipoprotein (apo) B-100 extracted from advanced human atherosclerotic lesions (26) supported the view that the MPO/hydrogen peroxide/chloride system converts LDL into an atherogenic form under in vivo conditions.

However, the cellular mechanisms leading to HOCl-LDL uptake/processing by macrophages has not yet been addressed. We, therefore, put major emphasis on the clarification of whether scavenger receptors on differentiated macrophages are responsible for HOCl-LDL uptake. A variety of scavenger receptors expressed on macrophages has been cloned; however, previous studies demonstrated that scavenger receptors class A, e.g. SR-A/I, and class B (e.g. CD36) are the principal receptors responsible for binding and uptake of modified LDL (for review see Refs. 27 and 28). Both type I and type II SR-A bind a diverse array of macromolecules, including bacterial surface lipids (endotoxin and lipoteichoic acid), β-amyloid fibrils, protein modified by advanced glycation (advanced glycation end products), and modified lipoproteins, e.g. acetylated LDL (ac-LDL) or copper-oxidized-LDL (Cu-ox-LDL), respectively. In vivo CD36 is involved in diverse processes as recognition of senescent or apoptotic cells, fatty acid transport, cell-matrix interaction, and antiangiogenic actions. CD36 mediates lipid accumulation and macrophage foam cell formation in vitro and in vivo. SR-BI, another class B (type I) scavenger receptor, has a multiligand specificity for various forms of native and modified (lipo)proteins (for review, see Ref. 29). Although SR-BI in comparison to SR-A and CD36 is less abundant in atherosclerotic lesions, its mRNA expression pattern during differentiation of human macrophages is similar to SR-AI and CD36, and both Cu-ox-LDL and ac-LDL may up-regulate its expression (30). Previous studies suggested that the primary routes for entry of ligands by macrophages are coated pits or caveolin-dependent endocytosis and/or phagocytosis. However, ligands can also be internalized non-concentration-dependent via fluid-phase uptake. Larger pinosomes termed macropinosomes can also internalize fluid-phase macromolecules via macropinocytosis (31).

We here provide evidence that HOCl-LDL is internalized by receptor-mediated endocytosis via class B scavenger receptor CD36 and SR-BI and partly via a mechanism resembling phagocytotic ingestion. At physiologically relevant HOCl concentrations HOCl-LDL is not recognized by SR-AI.
performed in triplicate. Results are expressed in µg of amino acids of 345 µg total LDL protein. Only those amino acids prone to be modified by HOCl were listed. REM, relative electrophoretic mobility; ND, not detectable.

| Amino Acid | 100:1 | 200:1 | 400:1 | 800:1 |
|------------|-------|-------|-------|-------|
| Cys        | 0.7   | ND    | ND    | ND    |
| Met        | 3.1   | ND    | ND    | ND    |
| Tyr        | 17.4  | 16.5  | 15.4  | 14    |
| Lys        | 31.4  | 29.9  | 28.1  | 26.2  |
| His        | 10.5  | 10.2  | 10.0  | 9.4   |
| Arg        | 15.7  | 15.5  | 15.5  | 15.5  |
| REM        | 1     | 1.15  | 1.35  | 1.75  | 2.1   |

Cell Culture Studies

Characterization of native and HOCl-modified LDL

Amino acid analysis was performed as described under “Materials and Methods.” Data shown represent mean values from one experiment performed in triplicate. Results are expressed in µg of amino acids of 345 µg total LDL protein. Only those amino acids prone to be modified by HOCl were listed. REM, relative electrophoretic mobility; ND, not detectable.

| Amino Acid | 100:1 | 200:1 | 400:1 | 800:1 |
|------------|-------|-------|-------|-------|
| Cys        | 0.7   | ND    | ND    | ND    |
| Met        | 3.1   | ND    | ND    | ND    |
| Tyr        | 17.4  | 16.5  | 15.4  | 14    |
| Lys        | 31.4  | 29.9  | 28.1  | 26.2  |
| His        | 10.5  | 10.2  | 10.0  | 9.4   |
| Arg        | 15.7  | 15.5  | 15.5  | 15.5  |
| REM        | 1     | 1.15  | 1.35  | 1.75  | 2.1   |

Dependently up to 20 and 10%, respectively, at the highest HOCl concentrations. Whereas the ε-amino group of arginine is not prone to be modified, the ε-amino group of lysine represents a preferential target for HOCl attack. The increase in the anionic charge of LDL, a result from modification of the ε-amino group of peptidyl lysine residues from apoB-100, is further reflected by an increased electrophoretic mobility of the modified LDL particle. In line with previous reports (23) no significant increase of lipid peroxidation products was detected as a function of increasing oxidant:LDL molar ratio ranging between 100:1 and 800:1 (data not shown).

Based on in vitro experiments HOCl concentrations at sites of acute inflammation were calculated to be in the range of 340 µM or above (45, 46). Assuming LDL plasma concentrations of ~2 µM (and it is conceivable that subendothelial concentrations could be lower) one would yield a minimal estimate HOCl:LDL molar ratio of ~200:1. Therefore, an oxidant:LDL molar ratio of 100:1 up to 400:1 was considered suitable for further experiments. Previous reports suggest that exposure of LDL to reagent HOCl results in LDL aggregation due to the formation of chloramines from apoB-100 lysine amino groups (23, 47). Because aggregation could be reversed by the addition of methionine or lysine (leading to reversion of apoB-100 chloramines), we studied whether HOCl-LDL aggregates are stable in culture medium (RPMI). Therefore, native and HOCl-LDL (1 mg of protein/ml of phosphate-buffered saline, oxidant:LDL molar ratio 400:1) were dialyzed against phenol red-free RPMI and analyzed by dynamic light scattering experiments as described (35). Under these conditions no HOCl-LDL aggregates were detected to be present in RPMI. The hydrodynamic radius of HOCl-LDL only moderately increased from 12.7 ± 1.3 to 16.6 ± 2.8 nm, indicating that no aggregation occurs under the conditions applied.

Binding and Cell Association of HOCl-LDL to Macrophages

To investigate binding properties of HOCl-LDL to THP-1 cells at 4 °C, the protein moiety of the lipoprotein particle was labeled with 125I Na. Non-linear regression analysis revealed a significant increase in binding affinity (Kd values) and saturable binding characteristics for HOCl-LDL when compared with native LDL (Fig. 1A, Table II). This indicates that HOCl-LDL is recognized by receptor(s) or binding protein(s). THP-1 macrophages were further incubated with HOCl-LDL for 24 h, and cellular cholesterol content was estimated. Incubation of cells led to a significant accumulation of free (Fig. 1B) and esterified cholesterol from HOCl-LDL (Fig. 1C). It is important to note that under the conditions described LDL only marginally increased cellular CE content (Fig. 1C). In contrast to binding experiments at 4 °C, cell association of HOCl-LDL at 37 °C is not saturable (Fig. 1D), indicating involvement of a pathway...
resembling phagocytic ingestion. Therefore, cell experiments were performed in the presence of cytochalasin D, a microfilament-disrupting agent (48) known to block phagocytosis and macropinocytosis. About 35–46% of cell association of LDL and HOCl-LDL (at concentrations higher than 100 g of protein/ml), respectively, could be inhibited by cytochalasin D (Fig. 1D). Association of vortex-aggregated LDL, which is internalized through a phagocytic mechanism (49), was inhibited by 90% by cytochalasin D treatment of cells (data not shown). Previous findings revealed that modified LDL particles are internalized by macrophages in part via macropinocytosis, a phagocytic mechanism that leads to ingestion of fluid-filled macropinosomes (50). Therefore, we were interested to what extent fluid-phase endocytosis (measured using [3H]sucrose) contributes to the internalization of HOCl-LDL. Fluid-phase endocytosis accounted for 27% (LDL) and 18% (HOCl-LDL) of the total [3H]CE lipoprotein uptake by THP-1 macrophages at the highest lipoprotein concentrations tested (500 g/ml) (Table III). At this lipoprotein concentration, cytochalasin D inhibited 35% (LDL) and 44% (HOCl-LDL) of the lipoprotein uptake (Fig. 1D), and therefore, fluid-phase endocytosis accounted for 70 and 53% of cytochalasin D-inhibitable phagocytic ingestion of LDL and HOCl-LDL, respectively. Of note, the presence of different concentrations of LDL or HOCl-LDL did not interact with [3H]sucrose ingestion by THP-1 cells.

Next, we investigated whether HOCl-LDL-associated lipids are delivered to THP-1 macrophages by a selective uptake mechanism. During this process, commonly mediated by SR-BI lipids are internalized by the cell without concomitant lipoprotein particle endocytosis. To compare the efficiency of CE-uptake, HOCl-LDL was labeled in the protein, and the lipid moiety and the cell association of both tracers was measured. For HOCl-LDL, apparent [3H]CE uptake by THP-1 cells was similar to holoparticle association (sum of associated and de-
Differentiated THP-1 macrophages were incubated for 4 h with 100 and 500 μg of protein/ml of LDL or HOCl-LDL (oxidant:lipoprotein molar ratio of 400:1) in the presence of 1 μCi/ml [3H]Sucrose. In a parallel experiment THP-1 cells were incubated with 100 and 500 μg of protein/ml of [3H]CE-LDL or [3H]CE-HOCl-LDL for the same time period. The amount of fluid-phase ingestion by macrophages was calculated by dividing the cell-associated radioactivity ([14C]Sucrose) by total radioactivity ([14C]Sucrose) added to the medium. The fluid-phase uptake of labeled lipoproteins was calculated by multiplying macrophase fluid endocytosis (μl of medium/mg of cell protein) and the [3H]Lipoproteins added to the medium. To calculate the percentage of fluid phase from total lipoprotein uptake, the fluid-phase uptake of [3H]CE lipoproteins was divided by the total uptake of [3H]CE lipoproteins and given on a percentage basis.

| LDL | Fluid phase uptake | LDL uptake | Fluid phase | Total | Fluid phase/Total |
|-----|-------------------|------------|-------------|-------|------------------|
| μg/ml | μg/mg of cell protein/2 h | ng of protein/mg of cell protein | % |
| 100 | 0.83 ± 0.1 | 83 ± 0.1 | 748 ± 30 | 11.1 |
| 500 | 0.83 ± 0.1 | 415 ± 0.5 | 1522 ± 67 | 27.2 |

| HOCl-LDL | Fluid phase uptake | HOCl-LDL uptake | Fluid phase | Total | Fluid phase/Total |
|---------|-------------------|-----------------|-------------|-------|------------------|
| μg/ml | μg/mg of cell protein/2 h | ng of protein/mg of cell protein | % |
| 100 | 0.83 ± 0.1 | 83 ± 0.1 | 983 ± 62.7 | 8.5 |
| 500 | 0.83 ± 0.1 | 415 ± 0.5 | 2258 ± 114 | 18.3 |

Table III: Contribution of fluid-phase endocytosis to total HOCl-LDL uptake by THP-1 cells

Differentiated THP-1 macrophages were incubated for 4 h with 100 and 500 μg of protein/ml of LDL or HOCl-LDL (oxidant:lipoprotein molar ratio of 400:1) in the presence of 1 μCi/ml [3H]Sucrose. In a parallel experiment THP-1 cells were incubated with 100 and 500 μg of protein/ml of [3H]CE-LDL or [3H]CE-HOCl-LDL for the same time period. The amount of fluid-phase ingestion by macrophages was calculated by dividing the cell-associated radioactivity ([14C]Sucrose) by total radioactivity ([14C]Sucrose) added to the medium. The fluid-phase uptake of labeled lipoproteins was calculated by multiplying macrophase fluid endocytosis (μl of medium/mg of cell protein) and the [3H]CE-labeled lipoproteins added to the medium. To calculate the percentage of fluid phase from total lipoprotein uptake, the fluid-phase uptake of [3H]CE lipoproteins was divided by the total uptake of [3H]CE lipoproteins and given on a percentage basis.

Interaction of HOCl-LDL with Scavenger Receptors on THP-1 and CHO Cells

Because binding studies at 4 °C revealed that binding of HOCl-LDL to THP-1 macrophages is saturable and specific we were interested in obtaining information about the recognition site(s) for HOCl-LDL on these cells. For these studies competition experiments with THP-1 cells were performed followed by binding studies of HOCl-LDL to CHO cells overexpressing candidate scavenger receptors.

Competition Studies—Previous studies demonstrate that scavenger receptor class A-II and CD36 are the principal receptors responsible for the binding and uptake of modified low density lipoproteins on macrophages (53). Because SR-AI/II has been reported to mediate binding and degradation of ac-LDL, competition experiments were performed using [3H]CE-ac-LDL as a ligand. At 37 °C unlabeled ac-LDL was observed to inhibit 80% of the cell association of [3H]CE-ac-LDL by THP-1 cells when present in 20-fold excess (Fig. 3A). Neither HOCl-LDL nor native LDL exhibited remarkable displacement capacity for cell association of [3H]CE-ac-LDL. However, HOCl-LDL strongly inhibited the cell association of moderately Cu-ox-LDL (Fig. 3B), a known ligand for class B scavenger receptors (39). Next, we were interested to what extent Cu-ox-LDL competes for HOCl-LDL binding. Most importantly, Cu-ox-LDL had the same efficiency as HOCl-LDL to block [3H]CE-HOCl-LDL cell association to THP-1 cells (Fig. 3C). Therefore, it is reasonable to assume that HOCl-LDL binds to the same THP-1 receptors/binding proteins as does Cu-ox-LDL.

SR-AI—Charge-neutralizing derivatization of lysine amino groups on apoB-100 of LDL is regarded as critical for recognition of modified forms of LDL by SR-AI/II (54). When LDL was exposed to increasing HOCl concentrations the percentage of free lysine groups on apoB-100 was decreased by 15%, no inhibition was observed with HOCl-LDL under the same conditions (graph not shown). We, therefore, conclude that endogenously synthesized lipoprotein lipase does not contribute to HOCl-LDL uptake.

CD36—To assess whether CD36 is involved in HOCl-LDL uptake, the next series of experiments was performed with CHO[hCD36] cells. Cell association studies showed that HOCl-LDL binds to CD36 with similar capacity as did Cu-ox-LDL, and the cell association of both HOCl-LDL and Cu-ox-LDL could be blocked by anti-C36 antibody (Fig. 5A). Binding studies at 4 °C (Table II) and association experiments at 37 °C (Fig. 5B) revealed that even a low degree of HOCl modification (oxidant:LDL molar ratio of 100:1) leads to enhanced recognition by CD36-overexpressing cells. In addition the binding affinity to CD36 was significantly increased as a function of increased HOCl:LDL molar ratio (Table II). Holoparticle association and degradation experiments revealed that LDL modified at a low oxidant:LDL molar ratio (100:1) binds to CD36 but is apparently not internalized because the same degradation rates of HOCl-LDL in the absence or presence of a CD36-blocking antibody were measured (Fig. 5C). In contrast, when LDL is modified by an oxidant:LDL molar ratio of 400:1 the modified LDL particle is internalized and degraded by...
CHO[hCD36] cells. Results shown in Fig. 5, A and B, confirm previous findings with CD36-overexpressing COS cells that human LDL does not bind to CD36 (39).

SR-BI—Because SR-BI is expressed on differentiated THP-1 macrophages (55) and SR-BI shares comparable ligand specificity with CD36, we were interested in whether SR-BI might contribute to binding and uptake of HOCl-LDL. To clarify the role of SR-BI as a candidate receptor for HOCl-LDL, binding experiments were performed with ldlA[mSR-BI] cells. Similar as found with CHO[hCD36] cells, HOCl-LDL is recognized by ldlA[mSR-BI] cells, and ligand affinity to SR-BI increases as a function of an increasing degree of HOCl modification (Fig. 6, Table II). However, compared with experiments performed with CHO[hCD36] cells, the binding affinity of HOCl-LDL to SR-BI-overexpressing cells is increased (Table II), and even LDL modified at a low oxidant:LDL molar ratio (100:1) is internalized and degraded by SR-BI (Fig. 6B).

Binding of HOCl-LDL to CD36 and SR-BI Is Not Mediated by the Lipid Moiety

Recent studies suggest a central role for oxidized phospholipids in recognition of ox-LDL by CD36 (56, 57). During these studies Cu-ox-LDL and LDL oxidized by the MPO/hydrogen peroxide/nitrite system were used, and binding to CD36 could be effectively inhibited by ox-LDL lipid extracts. In accordance the lipid moiety of Cu-ox-LDL efficiently reduced the association of HOCl-LDL to CD36 (Fig. 7A) and SR-BI (Fig. 7B) by >70%. In contrast, lipids isolated from LDL and HOCl-LDL exhibited a minimal inhibitory effect on HOCl-LDL binding. These results suggest that binding of HOCl-LDL to CD36 and SR-BI is mediated by modifications in the apolipoprotein domain.

DISCUSSION

HOCl-modified apoB-100 is present in human atherosclerotic lesions, and HOCl-modified (lipo)proteins are predominantly cell-associated and present in monocytes and macrophages (7, 26, 36). Despite intense interest in the role of HOCl in inflammatory injury and vascular disease, including recognition of HOCl-LDL by macrophages (23, 47) at physiologically relevant HOCl concentrations, information on specific binding properties and corresponding receptors mediating HOCl-LDL uptake is lacking.
Here we could show that binding of HOCl-LDL (at 4 °C) to differentiated THP-1 macrophages occurs through a high affinity, saturable, and specific interaction that is characteristic for a receptor-mediated process (Fig. 1A, Table II). Competition studies revealed that HOCl-LDL strongly competes for the uptake of Cu-ox-LDL (a ligand to scavenger receptors class B) (58) but did not inhibit the uptake of ac-LDL (a ligand to scavenger receptor class A). Experiments with CHO cells expressing CD36 and SR-BI confirmed that HOCl-LDL is a high affinity ligand for these receptors. Already an oxidant:LDL molar ratio of 100:1 led to recognition of HOCl-LDL by both receptors; however, efficient degradation via CD36 occurred at oxidant:LDL molar ratios higher than 200:1. A major finding of the present study is that the lipid moiety of HOCl-LDL does not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrated that the lipid moiety of HOCl-LDL could not contribute to the recognition of the modified lipoprotein particle by class B scavenger receptors. Cell culture experiments demonstrating that the lipid moiety of HOCl-LDL could not compete for binding of HOCl-LDL to CD36 and SR-BI. This observation is supported by the fact that HOCl (at low oxidant:LDL molar ratio) preferentially oxidizes apoB-100 with little or no lipid peroxidation (23, 59, 60) and by the fact that α-tocopherol is not depleted and that LDL cholesterol in HOCl-LDL is...
not oxidized (23, 60). The major type of primary oxidation products in LDL treated with HOCl at an oxidant:LDL (in terms HOCl:apoB-100) molar ratio of 400:1) were chloramines (23).

Treatment of LDL with HOCl may cause the lipoprotein to aggregate (47). As aggregation could be reversed by the addition of methionine or lysine or inhibited by reductive methylation of LDL lysine residues, chloramines are required for this process. Exposure of mouse peritoneal macrophages to HOCl-LDL (oxidant:lipoprotein molar ratio of 400:1) led to massive accumulation of cellular cholesterol and CE (23) in 2 h in the absence or presence of total lipids isolated from LDL, HOCl-LDL (400:1), and Cu-ox-LDL. Competitors, i.e. total lipid fraction (containing 200 μg of cholesterol/ml) isolated from native and modified lipoproteins, were added. The specific cell association of [3H]CE-labeled HOCl-LDL was determined as described under “Experimental Procedures.” Values are the averages of triplicate determinations from one representative experiment of two, and error bars represent the ranges of the measurements.

Fig. 7. Inhibition of [3H]CE-labeled HOCl-LDL cell association by lipids. [3H]CE-labeled HOCl-LDL (oxidant:LDL molar ratio of 400:1) was incubated at a protein concentration of 20 μg/ml with CHO(mCD36) cells (A) and LDL-A/SR-BI cells (B) at 37° C for 2 h in the absence or presence of total lipids isolated from LDL, HOCl-LDL (400:1), and Cu-ox-LDL. Competitors, i.e. total lipid fraction (containing 200 μg of cholesterol/ml) isolated from native and modified lipoproteins, were added. The specific cell association of [3H]CE-labeled HOCl-LDL was determined as described under “Experimental Procedures.” Values are the averages of triplicate determinations from one representative experiment of two, and error bars represent the ranges of the measurements.

In contrast to binding experiments at 4° C, the uptake of the HOCl-LDL at 37° C was not saturable, which indicates involvement of a phagocytic pathway. Phorbol myristate acetate has been found to stimulate macrophagocytosis in macrophages, a fluid-phase endocytic pathway that leads to formation of large vacuoles (>1 μm) from plasma membrane ruffles that envelop extracellular fluid (61, 62). Because THP-1 monocytes were differentiated by phorbol myristate acetate, macrophagocytosis could possibly contribute to the uptake of HOCl-LDL by THP-1 macrophages. This assumption is supported by observations that both ac-LDL and Cu-ox-LDL, respectively, are internalized by macrophages in part via macrophagocytosis (50). Macrophagocytosis can mediate the uptake of modified LDL by adsorptive endocytosis of lipoproteins bound to macrophagosome membranes and in part by the uptake that occurs via bulk phase-fluid ingestion of macrophagosomes. During the present study we have observed that about 40% of the THP-1 cell-mediated HOCl-LDL uptake can be inhibited by cytochalasin D; this supports the notion that HOCl-LDL uptake might also arise from non-scavenger receptor-mediated pathways. Whether HOCl-LDL binds to macrophagosome membranes of THP-1 macrophages and to what extent phorbol myristate acetate, known to alter macrophagocytosis (44), contributes to this endocytosis route of HOCl-LDL is currently under investigation.

Scavenger receptor family members SR-A (SR-AI/II) and CD36 have been identified as receptors for modified lipoproteins on macrophages, and their relevance to lipid uptake has been demonstrated in vitro and in vivo (53). Studies with SR-A or CD36 knockout mice revealed that disruption of either pathway partially blocks uptake of ac-LDL or Cu-ox-LDL in macrophages and retards atherosclerotic progression in hypercholesterolemic mice (63, 64). Expression of SR-AI/II is induced during monocyte to macrophage differentiation, and SR-AI/II mediates ~80% of the uptake of ac-LDL (65), a process that is not regulated by cellular cholesterol levels. The lack of recognition of HOCl-LDL by SR-AI is consistent with a relatively low level of oxidized apoB lysine at low oxidant:LDL molar ratios (Table I). Here we provide direct evidence, i.e. competition experiments on THP-1 cells (Fig. 3, B and C), in binding studies to CHO cells overexpressing CD36 in the absence or presence of anti-CD36 antibodies (Fig. 5A) that CD36 mediates specific ligand interaction with HOCl-LDL.

SR-BI, the prime receptor mediating selective lipid uptake (29), is highly expressed in the steroidogenic tissues, the liver, and placental cells, consistent with a role in mediating selective CE uptake. In parallel, SR-BI contributes to cellular cholesterol efflux from peripheral tissues, the first step during reverse cholesterol transport. SR-BI is also present in monocyte/macrophages including THP-1 cells (30), where it could function as a true ox-LDL receptor by displaying many of the features characteristic for classical scavenger receptors including uptake and degradation of oxidized lipoproteins (66). Braun and co-workers (67) report that the loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. The fact that HOCl-LDL is a high affinity ligand for SR-BI could lead to blockage of this receptor in a manner similar as shown for HOCl-HDL (36).

The full range of scavenger receptor functions is far from being clear, and in addition to their role in lipid uptake, these proteins have been shown to be involved in cellular adhesion and innate immune responses. Endothelial cells do represent the physiological target for adhesion, arrest, and transmigration of cells and proteins. HOCl-modified epitopes are abundantly present in situ, and endothelial cells bind and internalize MPO (68) and HOCl-modified lipoproteins (43), leading to impairment of endothelial function (19). Both MPO and HOCl-modified epitopes are abundantly present in various tissues under inflammatory conditions (7, 23, 69–72). Alternatively,
the presence of HOCl-modified epitopes in restricted areas of the placenta suggested local stimulation of the maternal immune system (73). This could be in line with a proposed function of chloramines to act as a bridge between inflammation and the immune system and probably between the afferent branches of the innate and acquired immune system (74). Scavenger receptors expressed by myeloid cells (macrophages and dendritic cells) and certain endothelial cells are the primary targets for uptake and clearance of effete components, such as modified host molecules and apoptotic bodies, to induce further intracellular signaling cascades after interaction of the respective ligand with its candidate scavenger receptor.

Acknowledgments—We are grateful to Monty Krieger (MIT, Cambridge, MA) for providing control and scavenger receptor overexpressing cells (CHO[mSR-AI] and ldlA[mSR-BI]) and Marsha Penham for detailed protocols of cell culturing. We thank Dr. Innerlohinger (Institute of Chemistry, University Graz, Austria) for performing dynamic light scattering experiments, Dr. Vardon (Department of Blood Transfusion, University Graz, Austria) for providing human plasma, and Cornelia Reinprecht for technical assistance.

REFERENCES

1. Chisolm, G. M., III, Hazen, S. L., Fox, P. L., and Cathcart, M. K. (1999) Redox Rep. 4, 183–187
2. Luiss, A. J. (2000) Nature 407, 233–241
3. Kettle, A. J., and Winterbourn, C. C. (1997) J. Clin. Invest. 100, 887–895
4. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) J. Biol. Chem. 273, 27499–27502
5. Hazen, S. L., and Heinecke, J. W. (1997) Circ. Res. 80, 583–588
6. Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M., and Krieger, M. (1994) J. Biol. Chem. 269, 7653–7658
7. Hammarqvist, F., Graebe, U., Pecile, P., Takeda, K., and Akerstrom, B. (1995) J. Biol. Chem. 270, 14059–14064
8. Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M., and Krieger, M. (1995) J. Biol. Chem. 270, 763–767
9. Acton, S., Lusher, P., Edelberg, J. M., and Groenewoud, E. F., and Malle, E. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 1654–1658
10. Bilder, D., Trigatti, B. L., Post, M. J., Sato, K., Simons, M., Edelberg, J. M., Braun, A., and Takeuchi, K. (2002) J. Biol. Chem. 277, 27954–27960
11. Krieger, M. (1999) J. Clin. Invest. 103, 1823–1833
12. Poussin, C., Poti, M., Carpenter, J. L., and Pugin, J. (1998) J. Biol. Chem. 273, 28254–28260
13. Shemer, R., and Averette, R. E. (1999) J. Biol. Chem. 274, 777–781
14. Cheung, A. K., and Groenewoud, E. F., and Malle, E. (2002) J. Biol. Chem. 277, 27961–27970
15. Najmaei, M., Yaar, M., and Krieger, M. (1998) J. Biol. Chem. 273, 7633–7638
16. Hazen, S. L., and Heinecke, J. W. (1997) Circ. Res. 80, 583–588
17. Hazen, S. L., and Heinecke, J. W. (1997) Circ. Res. 80, 583–588
18. Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M., and Krieger, M. (1994) J. Biol. Chem. 269, 7653–7658
19. Nuszkowski, A., Graebe, U., Pecile, P., Takeda, K., and Akerstrom, B. (1995) J. Biol. Chem. 270, 14059–14064
20. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
21. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
22. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
23. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
24. Malle, E., Waeg, G., Sattler, W., and Groenewoud, E. F., and Malle, E. (2002) J. Biol. Chem. 277, 27954–27960
25. Krieger, M. (1999) J. Clin. Invest. 103, 1823–1833
26. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
27. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
28. Febbraio, M., Hajjar, D. P., and Silverstein, R. L. (2001) J. Clin. Invest. 108, 1485–1496
29. Krieger, M. (1999) J. Clin. Invest. 103, 1823–1833
30. Poussin, C., Poti, M., Carpenter, J. L., and Pugin, J. (1998) J. Biol. Chem. 273, 28254–28260
31. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
32. Hazen, S. L., and Heinecke, J. W. (1997) J. Clin. Invest. 99, 2089–2096
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275