Hypochlorite-modified Low Density Lipoprotein Inhibits Nitric Oxide Synthesis in Endothelial Cells via an Intracellular Dislocation of Endothelial Nitric-oxide Synthase*

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Dislocalization of Endothelial Nitric-oxide Synthase*

Oxide Synthesis in Endothelial Cells via an Intracellular

Hypochlorous acid/hypochlorite, generated by the myeloperoxidase/H2O2/halide system of activated phagocytes, has been shown to oxidize/modify low density lipoprotein (LDL) in vitro and may be involved in the formation of atherogenic lipoproteins in vivo. Accordingly, hypochlorite-modified (lipo)proteins have been detected in human atherosclerotic lesions where they colocalize with macrophages and endothelial cells. The present study investigates the influence of hypochlorite-modified LDL on endothelial synthesis of nitric oxide (NO) measured as formation of citrulline (coproduct of NO) and cGMP (product of the NO-activated soluble guanylate cyclase) upon cell stimulation with thrombin or ionomycin. Pretreatment of human umbilical vein endothelial cells with hypochlorite-modified LDL led to a time- and concentration-dependent inhibition of agonist-induced citrulline and cGMP synthesis compared with preincubation of cells with native LDL. This inhibition was neither due to a decreased expression of endothelial NO synthase (eNOS) nor to a deficiency of its cofactor tetrahydrobiopterin. Likewise, the uptake of l-arginine, the substrate of eNOS, into the cells was not affected. Hypochlorite-modified LDL caused remarkable changes of intracellular eNOS distribution including translocation from the plasma membrane and disintegration of the Golgi location without altering myristoylation or palmitoylation of the enzyme. In contrast, cyclodextrin known to deplete plasma membrane of cholesterol and to disrupt caveolae induced only a disappearance of eNOS from the plasma membrane that was not associated with decreased agonist-induced citrulline and cGMP formation. The present findings suggest that mislocalization of NOS accounts for the reduced NO formation in human umbilical vein endothelial cells treated with hypochlorite-modified LDL and point to the important role of Golgi-localized NOS in these processes. We conclude that inhibition of NO synthesis by hypochlorite-modified LDL may be an important mechanism in the development of endothelial dysfunction and early pathogenesis of atherosclerosis.

Nitric oxide (NO) is generated in endothelial cells from the conversion of l-arginine to l-citrulline by the enzymatic action of an NADPH-dependent NO synthase (NOS) which requires tetrahydrobiopterin, FAD, and FMN as cofactors (1). Endothelial NOS (eNOS) is constitutively expressed and activated upon an increase of intracellular Ca2+. Following cell stimulation with agonists such as thrombin and bradykinin or through serine phosphorylation subsequent to cell stimulation with shear stress or insulin (2, 3). Optimal NO formation has been shown to be dependent on the availability of intracellular cofactors (tetrahydrobiopterin) (4, 5) and the subcellular localization of the enzyme (6). The eNOS is primarily membrane-bound and associated with Golgi membranes (7–9) and plasmalemmal caveolae (10–12) where it is quantitatively associated with caveolin, the structural coat component of these microdomains. The complex formation between eNOS and caveolin has been shown to inhibit enzyme activity, and the inhibitory effect was reversed upon binding of Ca2+/calmodulin (13–20). The membrane localization of eNOS is largely dependent on N-myristoylation and cysteine palmitoylation (cysteine 15 and 26) of the enzyme (7–12, 17, 21). Interestingly, inhibition of dual acylation or mutation of the palmitoylation sites leading to an attenuation of Golgi or caveole targeting, respectively, were both associated with an impaired cellular NO synthesis (7, 12).

Endothelium-derived NO exerts vasodilatory, growth regulatory, and antithrombotic activities thus being an important regulator of cardiovascular homeostasis (22). Evidence is accumulating that NO determines the anti-atherosclerotic properties of the endothelium (23). All major risk factors for atherosclerosis including hypercholesterolemia, hypertension, and smoking have been associated with impaired vascular NO synthesis (24). An increasing number of studies suggest that oxidized/modified low density lipoprotein (LDL), which is considered to play a key role in the development of atherosclerosis, may regulate the availability of NO (25). Oxidized LDL (ox-LDL) has been shown to inhibit the NO-mediated endothelium-dependent vasorelaxation (26–28). The underlying mechanisms are thought to involve the uncoupling of G protein-dependent signal transduction (29), an increased inactivation of NO (30–32), and a reduced formation of NO due to a limited availability of l-arginine (33) or a decrease in eNOS-mRNA transcription.
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stability and eNOS expression (34, 35). Recent findings suggest that inhibition of endothelial NO formation by oxLDL may also be related to an alteration of subcellular eNOS localization. Blair et al. (36) showed that an acute treatment of endothelial cells with oxLDL depleted caveolae of cholesterol and caused eNOS and caveolin-1 to translocate from the plasma membrane without affecting palmitoylation, myristoylation, or phosphorylation of the enzyme. Concomitantly, acetylcholine-induced activation of the enzyme was impaired.

Most studies investigating the effects of oxLDL used Cu2+-oxidized LDL as an experimental model. In contrast, there appears to be little evidence that free metal ions play an important role in the early development of atherosclerosis (25). The analysis of stable oxidation products in the human artery wall suggests that oxidation of LDL may rather involve the 15-lipoxygenase pathway, reactive nitrogen species, or myeloperoxidase-initiated reactions (25). Myeloperoxidase is released by activated neutrophils or monocytes and is catalyzing the reaction of H2O2 with Cl−, resulting in the formation of hypochlorous acid (HOCI) (37). This potent oxidant is thought to play an important role during microbial killing and to contribute to inflammatory tissue injury and atherogenesis. HOCI has been shown to oxidize LDL in vitro thereby generating a particle that caused foam cell formation (38), stimulation of neutrophil adherence to endothelial cells (39), production of reactive oxygen species by neutrophils (39), interleukin-8 formation by monocytes (40), and an increase in platelet aggregation (41). Several studies showed the presence of enzymatically active myeloperoxidase (42), HOCI-oxidized (lipo)proteins (43, 44), 3-chlorotyrosine (45) and dityrosine (46), both markers for HOCI-derived protein oxidation (47, 48), in human atherosclerotic lesions supporting the functional importance of the myeloperoxidase/H2O2/halide system as a potential in vivo oxidant. HOCI-modified epitopes have also been shown to colocalize with myeloperoxidase in lesion material including endothelial cells (44). Furthermore, HOCI-modified (lipo)proteins have been detected in inflammatory kidney tissues rich in myeloperoxidase (49).

In the present study we investigated the effects of hypochlorite-modified LDL on NO formation, eNOS protein and mRNA expression as well as subcellular localization of eNOS in human endothelial cells. We demonstrate that hypochlorite-modified LDL causes an inhibition of agonist-activated NO synthesis without altering the expression of eNOS, tetrahydrobiopterin availability, or arginine uptake into the cells. The reduced NO formation was associated with a striking intracellular redistribution of eNOS including the reduction of the enzyme in the plasma membrane and a disintegration of the perinuclear Golgi localization. Our data additionally underline the functional importance of Golgi-targeted eNOS since cycloexdrin that exclusively caused translocation of plasmalemmal eNOS did not inhibit cellular NO formation.

EXPERIMENTAL PROCEDURES

Materials—Plasticware for cell culture was from Greiner Labortechnik (Frickenhausen, Germany). Medium 199 (M199), human serum (HS), fetal calf serum, collagenase and human serum albumin (HSA) were from BioWhittaker Europe (Verviers, Belgium). t-1,2,3,5,6-[3H]Arginine monohydrochloride (61 Ci/mmol), [1-14C]ornithine monohydrochloride (303 mCi/mmol), [9,19-3H]palmitic acid (53 Ci/mmol), [9,19-3H]myristic acid (54 Ci/mmol), [14C]acetate, Megaprime DNA labeling system, [2-14C]AMP, ammonium salt (21 Ci/mmol), [14C]GMP Biotrak radiolabeled assay system, hyperfilm MP, hyperfilm p max, ECL, hyperfilm ECL, Hyperfilm+XL membranes, and PD-10 columns were purchased from Amersham Pharmacia Biotech. Tran32S-label (>1000 Ci/mmol) and methine-free RPMI medium were from ICN Biomedical Research Products (Costa Mesa, CA); EnHance solution was purchased from PerkinElmer Life Sciences; nicotinoylluciferin was from Millipore (Eschborn, Germany); specific antibodies against human eNOS (monoclonal, clone 3) and human caveolin-1 (monoclonal, clone C660, or rabbit polyclonal) were obtained from Transduction Laboratories (Lexington, KY); the monoclonal antibody against giantin was a kind gift of Dr. Hauri (University of Basel, Switzerland); the alkaline phosphatase anti-alkaline phosphatase complex (mouse monoclonal) and the bridging antibody rabbit anti-mouse IgG were purchased from Dako (Carpinteria, CA); the polyclonal antibody against human eNOS was from Santa Cruz Biotechnology (Santa Cruz, CA); and the fluorescein-labeled secondary antibodies (Cy-3- and Cy-2-labeled goat anti-mouse IgG and goat anti-rabbit IgG, suitable for multilabeling) were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). The protease inhibitor mixture Complete, EDTA-free, and an enzymatic test kit for cholesterol determination of cholesterol were purchased from Roche Molecular Biochemicals. Sepiapterin, t-nitroarginine methyl ester (t-NAME), and the cDNA probe for human eNOS were from Alexis Corp. (Laufelfingen, Switzerland). Endothelial cell growth supplement, peroxide-labeled anti-mouse IgG (Fab-specific), anti-mouse agarose, FAD, FMN, calmodulin, ionomycin, thrombin, EDTA, EGTA, trypsin/EDTA solution (0.05/0.02%, v/v), leupeptin, phenylmethylsulfonyl fluoride (PMSF), diithiobiotin (DTT), methyl-b-cyclodextrin and other reagents were from Sigma.

The composition of the Hepes buffer (pH 7.4) was as follows: 10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM glucose, 1.5 mM CaCl2. The solubilization buffer contained 100 mM NaOH, 2% Na2CO3, and 1% SDS. A protease inhibitor mixture stock solution was prepared by dissolving 1 tablet in 0.5 ml of 100 mM phosphate buffer (pH 7.0) and stored at −20 °C.

Cell Cultures—Human umbilical cord vein endothelial cells (HVEC) were prepared with 0.05% collagenase and cultured in 75-cm2 plastic flasks in M199 containing 15% fetal calf serum, 5% HS, and 7.5 μg/ml endothelial cell growth supplement. Confluent cultures were detached by trypsin/EDTA and plated on glass coverslips for immunohistochemical stainings, on 30 mm-diameter wells for the purpose of cGMP determination, on 60 mm-diameter dishes for the measurement of citrulline formation, [14C]arginine uptake, and incorporation of labeled compounds, and on 90 mm-diameter dishes for the investigation of the other parameters. Experiments were carried out with monolayers of the first to second passage.

Isolation and NaOCl Modification of Human LDL—Plasma from normolipemic human volunteers was collected in tubes containing 1 mg/ml EDTA. LDL (d 1.035–1.065 g/ml) was isolated by ultracentrifugation as described (50). LDL samples were stored under argon and used within 10 days following isolation. Prior to its modification LDL was desalted; low molecular mass compounds were removed by dialysis, and LDL was suspended in phosphate-buffered saline (PBS). The concentrations of LDL was determined spectrophotometrically using a molar absorption coefficient for OCI− of 350 cm−1 at 292 nm. One mg of LDL protein/ml of PBS was incubated (1 h, 4 °C, under argon) with NaOCl solution at molar ratios of NaOCl to LDL, ranging from 50 (100 μM NaOCl) to 400 (800 μM NaOCl) with the final pH adjusted to 7.4. The modified LDL preparations were passed over a PD-10 column to remove unreacted NaOCl. Characterization of NaOCl-modified LDL was revealed by increasing molar NaOCl/LDL ratio led to an increased relative electrophoretic mobility and a decreased percentage of free e-amino groups up to 75% as described previously (41).

Experimental Incubations—Preincubation of HUVEC with native LDL (nLDL) or NaOCl-modified LDL (NaOCl-LDL) suspended in Hepes buffer (pH 7.4) was performed in culture medium for 1–24 h. The indicated amounts of LDL used in the experiments were based upon the LDL protein concentration. If not otherwise announced, NaOCl-LDL modified at a molar NaOCl/LDL ratio of 400:1 was used. Neither nLDL nor modified LDL affected endothelial cell viability which was determined by trypan blue exclusion and ranged from 95 to 98% under the different conditions described. None of the tested native or modified lipoproteins altered the growth behavior of endothelial cells or their capability of protein synthesis as estimated by incorporation of [3H]leucine or [14C]labeled amino acids, respectively. Furthermore, no evidence of endothelial cell apoptosis was seen when nLDL or NaOCl-LDL were added to culture medium at the indicated concentrations and modifications, which was measured by the cell death detection enzyme-linked immunosorbent assay (Roche Molecular Biochemicals). Sepiapterin was dissolved in dimethyl sulfoxide (Me2SO). Stimulation of HUVEC was done by addition of ionomycin or thrombin. The concentration of lipoproteins was added to culture medium. The final concentration of Me2SO during experimental incubations and cell stimulation did not exceed 0.1%, and control cells received the same volume addition of solvent.

Immunohistochemical Detection of Hypochlorite-modified LDL in...
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**Endothelial Cells**—Endothelial cells cultured on coverslips were fixed for 10 min in 1% paraformaldehyde in PBS, washed with PBS, and permeabilized with methanol. Nonpermeabilized cells were used as a control. HOCl-modified epitopes were detected with the three-step alkaline phosphatase anti-alkaline phosphatase method (51). A hybridoma cell line supernatant (clone 2D10G9, 1:10) containing a monoclonal antibody that had been raised against HOCl-modified LDL (50) was used as a primary antibody. Nuclei were counterstained for 10 min with hematoxylin.

**Measurement of Citrulline Synthesis**—Citrulline synthesis was measured by a modification of a technique described previously (52). Briefly, HUVEC monolayers were incubated for 3 h with 20 µM cGMP, and labeled fragment of human eNOS-cDNA according to standard protocol. HOCl-modified LDL Inhibits Endothelial NO Synthesis

**Determination of cGMP**—HUVEC monolayers were incubated for 30 min in M199 containing 0.25% HSA and 0.5% isobutylmethylxanthine. Subsequently, the cells were stimulated with 10 µM t-arginine and 1 µM L-[3H]arginine. The arginine was counted after 15 min with ice-cold PBS containing 5 mM t-arginine and 4 mM EDTA, and the cells were denatured with 96% ethanol. After evaporation, the soluble cellular components were dissolved in 20 µM Hepes-Na (pH 7.5) and applied to 2-ml columns of Dowex AG50WX-8 (Na+ form). The radioactivity corresponding to the [3H]citrulline content of the eluate was quantified by liquid scintillation counting. Agostin-induced [3H]citrulline production was calculated from the difference in radioactivity from unstimulated and ionicomycin- or thrombin-stimulated cells and was expressed in pmoi/mg cell protein. Basal [3H]citrulline synthesis was determined from the L-NAMe (1 µM, 3 min preincubation)-inhibitable radioactivity in unstimulated cells and production was determined from the difference of cGMP content in cellular extract was measured by radioimmunoassay following the in-ethanol. When the ethanol had evaporated, 0.3 ml of buffer (50 mM Tris, balance, 0.025 mM pepstatin (pH 7.4)). For preparation of particulate and supernatant, cells were scraped into ice-cold Hepes/sucrose buffer (10 mM Hepes (pH 7.8), 250 mM sucrose, 1 mM EDTA containing 10 µl stock solution of protease inhibitor mixture/ ml) and sedimented by centrifugation at 14 000 × g for 10 min. The pellet was resuspended in 1 ml of the same buffer and homogenized 20 times with a tight Dounce homogenizer. The homogenate was layered on top of 12 ml of a 1–3 x linear sucrose gradient in 10 mM Hepes (pH 7.9) and centrifuged for 1.5 h at 40,000 × g and 4 °C in a Beckman SW-60 Ti swinging bucket rotor. The supernatant was then discarded from the top of the gradient, and fractions 2 and 3 containing cellular membranes were pelleted by centrifugation (100,000 × g, 1 h, 4 °C). The membranes were resuspended in buffer (20 mM Hepes, 10 mM potassium oxalate, 100 mM KCl, 10 mM MgCl2 (pH 7.6)) and layered over 3.5 ml of 40% sucrose solution in PBS as described by Enouf et al. (54). After centrifugation for 1.5 h at 95,000 × g and 4 °C in a swinging bucket rotor (Beckman SW-60 Ti), a band at the sample sucrose interface was recovered. This fraction was centrifuged for 1 h at 100,000 × g and 4 °C, resuspended in Hepes/sucrose buffer, and characterized as plasma membrane-enriched fraction by an increase of cholesterol content (566 ± 13 versus 329 ± 20 nmol/mg membrane protein in mixed membrane fractions) and of 5’-nucleotide activity (62 ± 5.8 versus 31 ± 3.7 mmol/mg membrane protein in mixed membrane fractions). Cholesterol was measured according to the cholesterol oxidase-sa-aminophenazone method using a test kit provided by Roche Molecular Biochemicals. The activity of the plasma membrane marker enzyme 5’-nucleotidase was determined according to Avruch and Wallach (55). Aliquots of the plasma membrane-enriched fraction were solubilized in Laemmli buffer and subjected to SDS-PAGE and immunoblotting as described above.

**Membrane Labeling and Immunoprecipitation of eNOS**—HUVEC monolayers were lysed with octyl glucoside buffer (50 mM Tris, 125 mM NaCl (pH 7.4), containing 60 µg octyl glucoside, 2 mM DTT, 0.05 mM EDTA, 1 mM PMSF, 0.6 µg leupeptin, and 0.025 µg pepstatin). Immunoprecipitation was performed with a monoclonal anti-eNOS antibody (50) that had been raised against HOCl-modified LDL (50). Hypochlorite-modified epitopes were detected with the three-step alkaline phosphatase anti-alkaline phosphatase method (51). A hybridoma cell line supernatant (clone 2D10G9, 1:10) containing a monoclonal antibody that had been raised against HOCl-modified LDL (50) was used as a primary antibody. Nuclei were counterstained for 10 min with hematoxylin.
RESULTS

Uptake of Hypochlorite-modified LDL into Endothelial Cells—HUVEC preincubated with 200 μg/ml NaOCl-LDL for 24 h in culture medium and labeled with a monoclonal antibody against HOCl-modified epitopes under permeabilizing conditions showed a strong granular staining throughout the cytoplasm (Fig. 1A). Since staining was not seen in nonpermeabilized cells preincubated with NaOCl-LDL (Fig. 1B), these data indicate an intracellular uptake of modified lipoproteins. HUVEC incubated with nLDL (200 μg/ml, 24 h) did not express HOCl-modified epitopes (Fig. 1, C and D).

Effect of Hypochlorite-modified LDL on Citrulline and cGMP Formation—NO production upon endothelial cell stimulation is accompanied by an increased synthesis of citrulline that is produced stoichiometrically with NO, and by an accumulation of intracellular cGMP that is generated when NO activates the soluble guanylate cyclase of the cells. Accordingly, both parameters indicate the formation of NO. Stimulation of HUVEC with ionomycin led to a [3H]citrulline formation of 150 ± 37 fmol/mg protein (n = 6) and a cGMP accumulation of 7.0 ± 0.9 pmol/mg protein (n = 6) (Fig. 2A). The response upon thrombin stimulation was generally lower (80 ± 15 fmol of [3H]citrulline/mg protein (n = 6) and 2.9 ± 0.1 pmol of cGMP/mg protein (n = 4)) (Fig. 2B). Pretreatment of HUVEC with nLDL (50–200 μg/ml, 24 h) had no effect on agonist-induced citrulline or cGMP synthesis (Fig. 2, A and B). NaOCl-LDL (50–200 μg/ml, 24 h preincubation with cells) inhibited citrulline production in comparison to nLDL in a concentration-dependent manner up to 39 (ionomycin-stimulation) or 73% (thrombin-stimulation) (Fig. 2, A and B). Similarly, agonist-induced cGMP formation was decreased in HUVEC pretreated with NaCl-modified LDL (up to 51 and 59% inhibition of ionomycin- and thrombin-stimulated cGMP accumulation, respectively, compared with control values in nLDL-treated cells) (Fig. 2, A and B). Neither nLDL nor NaOCl-LDL affected basal synthesis of [3H]citrulline and cGMP. Both agonist-induced citrulline and cGMP production in untreated HUVEC and in cells preincubated with nLDL or NaOCl-LDL were entirely blocked by a 30-min preincubation with 1 mM of the NOS inhibitor l-NAME (data not shown). The inhibitory effect of NaOCl-LDL on ionomycin-stimulated citrulline formation was time-dependent. After 6, 12, and 24 h preincubation of HUVEC with 200 μg/ml NaOCl-LDL, inhibition compared with nLDL-treated cells was 7, 15, and 42%, respectively (Fig. 3). A 1-h treatment of cells with NaOCl-LDL was not effective in inhibiting calcium-dependent citrulline formation.

Generally, the experiments were performed with NaOCl-LDL modified at a molar NaOCl:LDL ratio of 400:1. LDL modified at lower molar NaOCl:LDL ratios, however, had similar inhibitory effects on citrulline formation. Preincubation of HUVEC for 24 h with 200 μg/ml NaOCl-LDL modified at NaOCl:LDL ratios of 50:1, 100:1, 200:1, and 400:1 caused ionomycin-triggered [3H]citrulline production of 150 ± 24, 153 ± 7, 145 ± 4, and 130 ± 8 fmol/mg, respectively, whereas 220 ± 23 fmol/mg was measured in control cells treated with nLDL under identical conditions (n = 3).
FIG. 3. Time dependence of the effect of hypochlorite-modified LDL on ionomycin-induced citrulline formation. HUVEC were incubated with 200 μg/ml nLDL or NaOCl-LDL in culture medium for the indicated times. Cells were then stimulated in HEPES buffer (pH 7.4) with 2 μM ionomycin in the presence of 3.3 μCi/ml L-[3H]arginine for 15 min. The generated [3H]citrulline was separated from [3H]arginine by cation exchange chromatography and measured by liquid scintillation counting. Data are shown as agonist-induced increase in [3H]citrulline production calculated from the differences between stimulated and unstimulated cells (mean ± S.E., n = 2).

Effect of Sepiapterin on NaOCl-LDL-induced Inhibition of Citrulline Formation—To investigate whether the inhibitory effect of NaOCl-LDL on eNOS activity could be overcome by increasing the concentration of its cofactor tetrahydrobiopterin, incubations of HUVEC with lipoproteins were performed in the presence of sepiapterin which is intracellularly converted into tetrahydrobiopterin via a salvage pathway (56). Pretreatment of HUVEC with sepiapterin (10 μM, 24 h) led to a 2.5-fold increase in ionomycin-triggered citrulline formation that was not altered when nLDL was added in parallel (200 μg/ml, 24 h) (Table I). When HUVEC were coincubated with sepiapterin and NaOCl-LDL (200 μg/ml, 24 h), a similar inhibition of ionomycin-induced citrulline synthesis compared with nLDL-treated HUVEC as seen in cells without the addition of sepiapterin was observed (52 versus 51%, respectively) (Table I).

Effect of Hypochlorite-modified LDL on Arginine Uptake into Endothelial Cells—To determine whether NaOCl-modified LDL affects the transport of the NOS substrate arginine into the cells, HUVEC were incubated for 1–24 h in culture medium containing 335 μM L-[14C]arginine (3 mCi/mmol) in the presence of 200 μg/ml nLDL or NaOCl-LDL. Untreated control cells were incubated in parallel. Table II shows that the L-[14C]arginine uptake was time-dependent and was not altered by lipoproteins.

Effect of Hypochlorite-modified LDL on eNOS Expression and de Novo Synthesis—Northern blot analysis of RNA extracted from untreated endothelial cells and cells preincubated for 24 h with 200 μg/ml nLDL or NaOCl-LDL showed no difference in eNOS mRNA expression under the conditions examined (Fig. 4A). Accordingly, immunoprecipitation experiments comparing eNOS protein in 35S-labeled control HUVEC and cells preincubated with nLDL or NaOCl-LDL (200 μg/ml, 12-h preincubation and 3-h coincubation with Tran35S-label reagent) showed that the 35S-labeled eNOS band was not different between control and lipoprotein-pretreated cells, suggesting that eNOS de novo synthesis was not altered by nLDL or NaOCl-LDL (Fig. 4B). A comparable eNOS protein expression in cell lysates and subcellular fractions from untreated endothelial cells and cells incubated for 24 h with 200 μg/ml nLDL or NaOCl-LDL was confirmed in Western blotting studies. Fig. 4C shows that the eNOS protein was mainly located in the particulate fraction. Differences in eNOS expression were not seen in whole lysates or in particulate or cytosolic fractions between untreated and lipoprotein-treated cells.

Effect of Hypochlorite-modified LDL on Localization of eNOS—Localization of eNOS in control cells by indirect immunofluorescence revealed a strong crescent-shaped perinuclear staining, a faint fluorescence pattern diffusely distributed throughout the cell, and a discrete staining in the plasma membrane. Confocal microscopy showed a colocalization of eNOS with giantin, an integral protein of the Golgi membranes (57), and partially with caveolin-1 which was primarily localized at the leading edge and in the perinuclear region (Fig. 5, A and B). Pretreatment of HUVEC with nLDL (200 μg/ml, 24 h) did not affect eNOS distribution nor its colocalization with giantin and caveolin-1, respectively (Fig. 5, A and B). However, a marked intracellular redistribution of eNOS was observed after preincubating HUVEC with NaOCl-LDL (200 μg/ml, 24 h). Plasmaemmal staining of eNOS was considerably decreased, and the distinct granular staining in the perinuclear region was changed into a diffuse cytoplasmic fluorescence pattern. Additionally, colocalization with giantin or caveolin-1 was abolished or largely diminished, respectively.

Effect of Hypochlorite-modified LDL on the Association of eNOS and Caveolin-1 with a Plasma Membrane-enriched Subcellular Fraction—The NaOCl-LDL-induced mislocalization of eNOS was confirmed by Western blot experiments investigating...
ing a plasma membrane-enriched subcellular fraction prepared from control and lipoprotein-pretreated cells. Preincubation of HUVEC with NaOCl-LDL (200 µg/ml, 24 h) led to a reduction of eNOS in this membrane fraction, whereas no differences in eNOS expression were seen between untreated and nLDL (200 µg/ml, 24 h)-treated cells (Fig. 6). In contrast, caveolin-1 was similarly expressed in plasma membrane-enriched fractions from control and lipoprotein-pretreated cells (Fig. 6).

Effect of Hypochlorite-modified LDL on Coimmunoprecipitation of eNOS and Caveolin-1—Since NaOCl-LDL treatment of cells affected the translocation of eNOS but not of caveolin-1 from plasma membrane-enriched subcellular fractions, we tested whether the observed changes in eNOS localization were associated with alterations in eNOS-caveolin interactions. Fig. 7 shows that monoclonal antibodies against eNOS immunoprecipitated equal amounts of eNOS from lysates of control and nLDL (200 µg/ml, 24 h)-treated cells (Fig. 6). In contrast, caveolin-1 was similarly expressed in plasma membrane-enriched fractions from control and lipoprotein-pretreated cells (Fig. 6).

Effect of Hypochlorite-modified LDL on Acylation of eNOS—Because of the importance of fatty acylation for the intracellular compartmentalization of eNOS (7–12, 17, 21), we investigated whether the NaOCl-LDL-induced mislocalization of eNOS was related to an alteration of myristoylation or palmitoylation of the enzyme. HUVEC preincubated for 24 h with 200 µg/ml nLDL or NaOCl-LDL were radiolabeled with 

\[ ^3H \text{palmitate or } ^3H \text{myristate} \]

in the presence of lipoproteins. Fig. 8 shows that the steady-state palmitoylation or myristoylation of eNOS was not affected by treatment with either nLDL or NaOCl-LDL.

Effect of Hypochlorite-modified LDL on the Cholesterol Content of Mixed Membranes and Plasma Membrane-enriched Subcellular Fractions—We next analyzed whether the NaOCl-LDL-induced translocation of eNOS was related to changes in the cholesterol content of cellular membranes since plasma membrane and caveolar localization of eNOS have been shown to depend on membrane cholesterol (36, 58). Cholesterol was measured in mixed membrane fractions and plasma membrane-enriched subcellular fractions prepared from control cells and cells preincubated for 24 h with 200 µg/ml nLDL or NaOCl-LDL. As shown in Table III no differences in membrane cholesterol content were seen between control and lipoprotein-pretreated cells.

Effect of Cyclodextrin on the Localization of eNOS and the Formation of Citrulline and cGMP—To compare the NaOCl-
induced translocation of eNOS with a redistribution of eNOS that is caused by cholesterol depletion of plasma membranes, we treated cells with cyclohexim, a compound known to extract cholesterol from caveolae (59). Fig. 9 shows that the addition of cyclohexim (5 mM, 1 h) to the cells led to a disappearance of eNOS and caveolin-1 from the plasma membrane. However, in contrast to the changes seen after treatment of cells with NaOCl-LDL, the perinuclear localization of eNOS and its colocalization with caveolin-1 and giantin in the Golgi area were maintained.

Preincubation of HUVEC with cyclohexim (5 mM, 1 h) led to an increase of ionomycin-stimulated \(^{[3]H}\)citrulline formation from 188 ± 42 fmol/mg protein in untreated cells to 256 ± 47 fmol/mg (n = 3, not significant). Correspondingly, ionomycin-triggered cGMP formation was enhanced from 6.6 ± 0.5 pmol/mg protein to 9.2 ± 1.2 pmol/mg (n = 3, not significant).

The present study demonstrates that hypochlorite-modified LDL inhibits agonist-induced endothelial NO synthesis in a dose- and time-dependent manner. This was shown by a concomitant decrease of both the formation of citrulline, which is produced stoichiometrically with NO, and the accumulation of intracellular cGMP, which is generated upon NO-mediated activation of the soluble guanylate cyclase.

The effect of NaOCl-LDL on NO synthesis is likely to be related to an uptake of the particles into the cells since cell stimulation was performed in the absence of extracellular lipoproteins. Indeed, immunochemical findings of the present study show the occurrence of HOCl-modified epitopes in cells that had been incubated with NaOCl-LDL, and binding and internalization of hypochlorite-modified lipoproteins might have been mediated by scavenger receptors present on endothelial cells (58, 60–62). Hypochlorite-modification of LDL has already been demonstrated to impair endothelial-dependent arterial relaxation and NO synthesis in HUVEC (66, 67). At present, however, we have no evidence whether the lipid or the protein moiety of NaOCl-LDL or both are contributing to the impairment of NO synthesis observed in our study.

Several inhibitory mechanisms of oxLDL on NO synthesis reported in the literature (29, 33–35) could be excluded as responsible for the effects observed with hypochlorite-modified LDL. The present study shows the occurrence of HOCl-modified epitopes in cells that had been incubated with NaOCl-LDL, and binding and internalization of hypochlorite-modified lipoproteins might have been mediated by scavenger receptors present on endothelial cells (58, 60–62). Hypochlorite-modification of LDL has already been demonstrated to impair endothelial-dependent arterial relaxation and NO synthesis in HUVEC (66, 67). At present, however, we have no evidence whether the lipid or the protein moiety of NaOCl-LDL or both are contributing to the impairment of NO synthesis observed in our study.

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The present study demonstrates that hypochlorite-modified LDL inhibits agonist-induced endothelial NO synthesis in a dose- and time-dependent manner. This was shown by a concomitant decrease of both the formation of citrulline, which is produced stoichiometrically with NO, and the accumulation of intracellular cGMP, which is generated upon NO-mediated activation of the soluble guanylate cyclase.

The effect of NaOCl-LDL on NO synthesis is likely to be related to an uptake of the particles into the cells since cell stimulation was performed in the absence of extracellular lipoproteins. Indeed, immunochemical findings of the present study show the occurrence of HOCl-modified epitopes in cells that had been incubated with NaOCl-LDL, and binding and internalization of hypochlorite-modified lipoproteins might have been mediated by scavenger receptors present on endothelial cells (58, 60–62). Hypochlorite-modification of LDL has already been demonstrated to impair endothelial-dependent arterial relaxation and NO synthesis in HUVEC (66, 67). At present, however, we have no evidence whether the lipid or the protein moiety of NaOCl-LDL or both are contributing to the impairment of NO synthesis observed in our study.

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LDL in the present study. Our findings suggest that NaOCl-LDL acts downstream from signal transduction required for thrombin-induced NOS activation since NO formation was also reduced when NaOCl-LDL-treated cells were challenged with the calcium ionophore ionomycin. The effect of hypochlorite-modified LDL was not due to a deficiency of the NOS cofactor tetrahydrobiopterin since the inhibition of NO synthesis was measured even when the intracellular tetrahydrobiopterin levels were increased by coinubcation of cells with sepiapterin (56). Likewise, a deficiency of the NOS substrate L-arginine did not account for the observed effects because NaOCl-LDL did not alter the cellular uptake of this amino acid. Hypochlorite-modified LDL also did not affect de novo synthesis of NOS as measured in 35S-labeled cells. Accordingly, neither the amount of specific mRNA nor the expression of eNOS protein recognized with specific monoclonal antibodies in cell lysates were altered by pretreatment of cells with NaOCl-LDL. However, our findings suggest that the mechanism underlying the inhibition of agonist-stimulated NO formation by hypochlorite-modified LDL involves an alteration of the subcellular localization of eNOS. Immunofluorescence studies showed a distinct eNOS localization in the plasma membrane and in the Golgi region in control cells and nLDL-treated cells. In contrast, in endothelial cells preincubated with NaOCl-LDL eNOS was translocated from both compartments and revealed a diffuse cytoplasmic distribution. However, NaOCl-LDL did not alter the structure of the Golgi region as characterized by the Golgi marker protein giantin (57) and did also not lead to major changes of the distribution of caveolin-1 which is known to be colocalized in part with eNOS (9, 10). The results of the immunofluorescence microscopy were confirmed by subcellular fractionation data. Hypochlorite-modified LDL led to a reduction of eNOS association with a plasma membrane-enriched subcellular fraction, whereas caveolin-1 expression in this fraction was maintained. Interestingly, the overall membrane association of eNOS as shown by immunoblotting analysis of 100,000 × g membrane fractions was not modified by NaOCl-LDL. These findings confirm previous studies that demonstrated that a stable membrane association is not sufficient for proper membrane targeting (8) and further suggest a translocation of eNOS to a membrane compartment distant from plasma or Golgi membranes.

Our results are in line with previous studies demonstrating that NOS mislocalization is paralleled by an attenuated capacity for NO production in intact cells (7, 12). Different studies using cells transfected with myristoylation-deficient and/or palmitoylation-deficient eNOS mutants revealed that targeting of eNOS to caveolae or Golgi membranes is regulated in an acylation-dependent manner (7–12, 17, 21). In our experimental approach, however, translocation of eNOS from plasma membrane and Golgi was not associated with changes in the steady-state myristoylation or palmitoylation of eNOS, suggesting that additional mechanisms are involved in the control of the subcellular location of eNOS. It might be possible that hypochlorite-modified LDL leads to changes in the physico-chemical properties of membranes similar to that induced by oxLDL (68) or even gives rise to oxidative damage of certain cholesterol-rich membrane compartments. These alterations may lead to instabilities of eNOS interaction with proteins or lipids of Golgi or plasma membrane and mislocation to other membrane compartments. Reactive oxygen species, for example, have been shown to reduce the association of eNOS with caveolin-1 in caveolae-enriched membranes (69). A decreased interaction of eNOS with caveolin-1 as measured by coimmunoprecipitation experiments was also found after pretreatment of cells with NaOCl-LDL suggesting a disruption of the caveolin-eNOS regulatory cycle under these conditions and an eNOS movement independent of caveolin-1.

Recently, Blair et al. (36) and Uittenbogaard et al. (58) demonstrated that in addition to acylation the cholesterol content of caveolae is important for subcellular eNOS location and NO formation and may be a target for the regulation of NO synthesis by oxLDL. In these studies endothelial cells expressing eNOS exclusively in their plasma membrane were incubated for 1 h with Cu2+-oxidized LDL which led to a complete loss of caveolae-associated cholesterol and to a subsequent translocation of both eNOS and caveolin-1 from the plasma membrane to internal membranes (not Golgi or endoplasmic reticulum) (36). High density lipoproteins prevented the effects of oxLDL by preserving caveolar cholesterol content (58). The experimental design of these studies is different from our experimental ap.

Fig. 9. Effect of cyclodextrin on the localization of eNOS in endothelial cells. HUVEC on coverslips were incubated with 5 mM cyclodextrin in culture medium for 1 h. Subsequently, cells were washed, fixed, and labeled with the following antibodies. A, polyclonal anti-eNOS and monoclonal anti-giantin followed by Cy-3-labeled anti-mouse IgG and polyclonal anti-caveolin-1 followed by Cy-3-labeled anti-mouse IgG. B, monoclonal anti-eNOS and polyclonal anti-caveolin-1 followed by Cy-3-labeled anti-mouse IgG and Cy-2-labeled anti-rabbit IgG. Cells were observed with a laser scanning confocal microscope (Zeiss LSM 510). The bar represents 20 μm. A representative experiments out of five is shown.
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proach including the cellular model, the type of LDL modification, and the incubation time. Furthermore, in contrast to our study oxLDL was not internalized into the cells. Thus, although our findings agree with the studies by Blair et al. (36) and Uittenbogaard et al. (58) regarding the translocation of eNOS to internal membranes, the mechanisms underlying the eNOS redistribution may be different. Indeed, NaOCl-LDL treatment of cells did not alter the cholesterol content of a plasma membrane-enriched subcellular fraction, suggesting that the mislocalization of eNOS induced by hypochlorite-modified LDL cannot be attributed to cholesterol depletion. Moreover, our data reveal that cycloedrin, which had been shown to deplete the plasma membrane of cholesterol and to disrupt caveolae (59), induced a redistribution of eNOS that was different from the one seen after preincubation of cells with NaOCl-LDL. Cycloedrin caused a displacement of both eNOS and caveolin-1 from the plasma membrane, thus confirming that cholesterol is essential for eNOS incorporation into the plasmalemmal compartment (36, 58) but did not modify the perinuclear localization of eNOS that was clearly affected by NaOCl-LDL. Interestingly, despite the drastic effect of cycloedrin on the translocation of plasmalemmal eNOS, this compound did not inhibit agonist-induced endothelial NO formation. In contrast, agonist-induced NO synthesis in cycloedrin-treated cells was even slightly increased, which may probably be due to a dissociation of eNOS from inhibiting factors such as caveolin-1. Taken together, our data point to an important contribution of Golgi-located eNOS to overall NO production in HUVEC since an inhibition of cellular NO formation was only observed when both a disintegration of Golgi-located eNOS and a reduction of plasmalemmal eNOS occurred.

In summary, the present findings demonstrate that hypochlorite-modified LDL inhibits agonist-induced endothelial NO formation in a time- and concentration-dependent manner. The decreased NO formation was neither due to substrate nor cofactor deficiencies nor to changes in eNOS expression but was paralleled by an intracellular redistribution of eNOS. Our data confirm the importance of specific intracellular membrane targeting for eNOS activity and indicate that Golgi-located eNOS may significantly contribute to overall NO formation in HUVEC. Based on these in vitro findings we propose that hypochlorite-modified LDL may promote endothelial dysfunction in vivo thereby being involved in early atherogenic processes.

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Hypochlorite-modified LDL Inhibits Endothelial NO Synthesis

Hypochlorite-modified Low Density Lipoprotein Inhibits Nitric Oxide Synthesis in Endothelial Cells via an Intracellular Dislocalization of Endothelial Nitric-oxide Synthase

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