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Modification Induced by Homocysteine and Low-Density Lipoprotein on Human Aortic Endothelial Cells: An *In Vitro* Study

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The aim of the present study was to investigate the effect exerted by low-density lipoprotein (LDL) modified by homocysteine (Hcy)-thiolactone (Hcy-LDL) on functional properties on human endothelial cells. Hcy-thiolactone, a reactive product formed in human cells from enzymatic conversion of Hcy, was hypothesized to play an important role in Hcy-induced vascular damages. Using endothelial cultured cells [human aortic endothelial cells (HAEC)] as cellular model, we evaluated nitric oxide (NO) production, cytoplasmic Ca^{2+} levels, Na^+/K^+-ATPase activity, and peroxynitrite production in cells incubated in the presence of control LDL or Hcy-LDL. Homocysteinylation of LDL was carried out by incubation of LDL, isolated from plasma of healthy subjects, with 100 μM Hcy-thiolactone. A significant increase in cytoplasmic Ca^{2+} levels and peroxynitrite production and a decrease in Na^+/K^+-ATPase and NO production in HAEC incubated with Hcy-LDL compared with HAEC incubated with control LDL were observed. Moreover, a positive correlation was found between Na^+/K^+-ATPase activity and cytoplasmic Ca^{2+} content and between peroxynitrite activity and cytoplasmic Ca^{2+} content. In conclusion, our results demonstrated that LDL homocysteinylated *in vitro* induced alterations of functional properties and NO metabolism of human endothelial cells. (*J Clin Endocrinol Metab* 89: 4558–4561, 2004)

SEVERAL MECHANISMS HAVE been investigated to explain the role of homocysteine (Hcy) in the pathogenesis of vascular disease (1, 2). These mechanisms include endothelial injury, reduction of vascular nitric oxide (NO) production and bioavailability, a mitotic effect on smooth muscle cells, an influence on leukocyte behavior and hemostasis, and oxidative modification of low-density lipoprotein (LDL). Many of these mechanisms appear to be related to oxidative stress generated by the oxidation of thiols to disulfides, which causes reactions producing reactive oxygen species like superoxide, hydrogen peroxide, and hydroxyl radical (3, 4). Moreover, an inhibitory action of Hcy on platelet NO production has been observed *in vitro*, and it has been hypothesized that the platelet activation caused by a reduction in NO release could be involved in the Hcy-linked vascular damage (5).

Jakubowski (6) suggested that Hcy toxicity could be related to Hcy-thiolactone, a reactive product formed in human cells from enzymatic conversion of Hcy. In fact, it has been demonstrated *in vitro* that Hcy-thiolactone induces apoptosis and death in human endothelial cells causing vascular damages (7).

Moreover, several studies by us and others have demonstrated that plasma lipoproteins are susceptible to homocysteinylation and that the interaction between Hcy-thiolactone and amino groups of apo-B lysyl residues of LDLs induces the formation of LDL modified by Hcy-thiolactone (Hcy-LDL) adducts (6, 8–11). Homocysteinylation of LDL is accompanied by structural and functional alterations (12, 13); therefore, it has been suggested that homocysteinylation could increase the atherogenicity of LDL (12, 13).

This hypothesis is confirmed by our previous study that demonstrated, for the first time, that Hcy-LDL induced an increase in oxidative damage and a decreased viability on human endothelial cells (11).

Alterations of functions and oxidative damages and alteration of NO metabolism induced by interactions with modified LDL (oxidized or glycated LDL) on vascular endothelial cells (14, 15) and platelets (16) have been previously observed, and this has been suggested to represent the early stages of the development of atherosclerosis.

The aim of the present study was to further investigate the effect of Hcy-LDL on functional properties of human endothelial cells. Therefore, cytoplasmic Ca^{2+} levels, peroxynitrite production, Na^+/K^+-ATPase, and NO production in cultured human aortic endothelial cells (HAEC), incubated with control LDL (C-LDL) or Hcy-LDL, have been evaluated.

Materials and Methods

HAECs were purchased from Cambrex (Bergamo, Italy) and cultured in Endothelial Cell Medium-2 (EGM-2) supplemented with Low Serum Growth Supplement (Cambrex).

Preparation and characterization of LDL

Plasma was separated from freshly drawn human blood from 20 healthy male subjects without microvascular or macrovascular complications. The clinical parameters of subjects are reported in Table 1.

Plasma was prepared by centrifugation at 3000 rpm for 15 min and, thereafter, used for the preparation of LDL. LDLs (density between 1.025 and 1.063 g/ml) were isolated by single vertical spin gradient ultracentrifugation as described by Chung *et al.* (17). After dialysis at 4 C for 24 h

Abbreviations: $[Ca^{2+}]_i$, Intracellular Ca^{2+}; C-LDL, control low-density lipoprotein; DCF, 2,7-dichlorofluorescein; DCFDA, 2,7-dichlorofluorescein diacetate; HAEC, human aortic endothelial cell; Hcy, homocysteine; Hcy-LDL, low-density lipoprotein modified by homocysteine-thiolactone; LDL, low-density lipoprotein; NO, nitric oxide.

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TABLE 1. Clinical characteristics of subjects from whom LDL were obtained

	Healthy control subjects (n = 20)
Age (yr)	39 ± 8
BMI (kg/m^2)	22.5 ± 2.5
Fasting plasma glucose (mmol/liter)	5.0 ± 0.3
HbA_{1c} (%)	5.0 ± 0.4
Plasma LDL cholesterol (mmol/liter)	2.45 ± 0.16
Plasma HDL cholesterol (mmol/liter)	1.15 ± 0.06
Plasma triglycerides (mmol/liter)	1.35 ± 0.22

Means ± SD are shown. HbA_{1c}, Glycosylated hemoglobin; HDL, high-density lipoprotein.

against 10 mmol/liter PBS (pH 7.4), the protein concentration of LDL was determined by the method of Bradford (18). The concentrations of triglycerides, phospholipids, and cholesterol were determined as previously described (15). LDLs were sterilized on a 0.2-μm Millipore membrane (Millipore, Billerica, MA) before being incubated with HAEC.

The study was performed in accordance with the principles of the Declaration of Helsinki as revised in 1996, and informed consent was obtained from the study subjects.

Incubation of human LDL with Hcy-thiolactone

The *in vitro* homocysteinylation of LDL was carried out following the experimental conditions described in our previous study (11).

Briefly, an aliquot of LDL (100 μg of LDL protein) resuspended in 10 mmol/liter PBS (pH 8.2) was incubated at 37 C in the absence (C-LDL) or presence of Hcy-thiolactone (100 μmol/liter; Hcy-LDL) for 2 h. The mixture was incubated with gentle stirring for the indicated times and passed through a Sephadex G-25 column equilibrated with 10 mmol/liter PBS (pH 8.2) to separate the unreacted Hcy-thiolactone.

The reaction of homocysteinylation of LDL was verified by the study of the increase in sulfhydryl groups in Hcy-LDL with respect to control LDL (8, 9, 11).

The levels of sulfhydryl groups were assayed using the dithionitrobenzoic acid reagent (19). An aliquot of LDL (100 μg) that was treated in different experimental conditions was incubated with 0.25 mol/liter Tris-HCl (pH 8.2) and 20 mmol/liter EDTA in the presence of 0.1 mmol/liter dithionitrobenzoic acid and absolute methanol. After incubation for 20 min at room temperature, LDL were centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 412 nm. The levels of sulfhydryl groups were quantified using a stock solution of 1 mmol/liter glutathione reduced. The concentrations of sulfhydryl groups are given in terms of nanomoles per milligram of LDL protein.

Incubation of HAEC with LDL

At 80% confluence (~500,000 cells/ml), cultured HAEC were incubated for 3 h at 37 C with EGM-2 alone (control HAEC), with C-LDL (100 μg/ml in EGM-2), or with homocysteinylated LDL (Hcy-LDL 100 μg/ml in EGM-2), following the experimental conditions described in our previous study (11). After the incubation, the monolayers were removed by scraping and resuspended in fresh culture medium, and the following parameters were evaluated: NO and peroxynitrite production, cytoplasmic Ca^{2+} levels, and Na^+/K^+-ATPase activity.

NO production

NO levels were measured in the supernates of lysed cells, as described by Camilletti *et al.* (20), using the Griess reaction (21). Briefly, cells were suspended in NO buffer (HEPES 25 mM, NaCl 140 mM, KCl 5.4 mM, $CaCl_2$ 1 mM, and $MgCl_2$ 1 mM, pH 7.4) containing 1.44 mM reduced nicotinamide adenine dinucleotide phosphate and incubated for 1 h at 37 C after the addition of L-arginine 100 μM. The reaction was then stopped by freeze-thawing the sample, which was then sonicated. Each sample was incubated for 1 h at 37 C after that addition of nitrate reductase (20 mU), which reduces nitrate to nitrite. After centrifugation at 3000 rpm for 15 min, the supernatant was allowed to react with the Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, and 2.5% H_3PO_4). The chromophore absorption was read at 543 nm. Nitrite concentration was determined with sodium nitrite in water as standard. NO synthase activity was expressed in nanomoles per 10^6 cells.

Peroxynitrite production

Peroxynitrite production was studied by the fluorometric assay of 2,7-dichlorofluorescein (DCF) as described by Tannous *et al.* (22). Peroxynitrite can oxidize the nonfluorescent molecule DCF diacetate (DCFDA) to the fluorescent DCF.

DCFDA free base was prepared daily by mixing 0.05 ml of 10 mmol/liter DCFDA with 2 ml of 0.01 N NaOH at room temperature for 30 min. The mixture was neutralized with 18.0 ml of 25 mmol/liter PBS (pH 7.4). This solution was maintained on ice in the dark until use. The DCFDA-treated cells were incubated in NO buffer with L-arginine 100 μM for 15 min at 37 C in the dark room. The mixture was washed with PBS (pH 7.4) and then centrifuged for 2 min at 214 × *g*. The supernatant fluorescence was measured in a Perkin-Elmer MPF-66 spectrofluorometer (PerkinElmer, Wellesley, MA) at an excitation wavelength of 475 nm and emission wavelength of 520 nm.

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured in intact HAEC using the fluorescent probe Fura 2-AM, as previously described (23). Determinations were performed in a Perkin-Elmer MPF-66 spectrofluorometer at 37 C according to the method of Rao (24). Fluorescence intensity was read at a constant emission wavelength (490 nm), with changes in the excitation wavelength (340 and 380 nm). Calibration was carried out as described by Grynkiewicz *et al.* (25) with the following equation:

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}},$$

where K_d is the dissociation constant of the Ca^{2+}-Fura 2 interaction in the cytosolic environment; R is the ratio of the fluorescence intensities at excitation wavelengths 340 and 380 nm; R_{min} and R_{max} are the ratios of the fluorescence intensities without Ca^{2+} and with saturating levels of Ca^{2+}, respectively; and S_{f2} and S_{b2} are fluorescence intensities at 380 nm without Ca^{2+} and with saturating levels of Ca^{2+}, respectively. R_{min} and S_{f2} were measured after cellular lysis with 25% Triton X-100 and addition of 10 mM EGTA (pH 8.3). R_{max} and S_{b2} were determined after lysis and addition of 10 mM $CaCl_2$. Autofluorescence was subtracted before performing the Ca^{2+} calibration procedure.

Na^+/K^+-ATPase assay

The Na^+/K^+-activated Mg^{2+}-dependent ATPase activity was determined on HAEC by the Kitao and Hattori method (26). The ATPase activity was assayed by incubating 1 ml of HAEC (2×10^6 cells/ml) after sonication at 37 C in the reaction medium containing $MgCl_2$ (5 mM), NaCl (140 mM), and KCl (14 mM) in 40 mM Tris-HCl (pH 7.7). The ATPase reaction was started by the addition of 3 mM Na_2ATP and stopped 20 min later by the addition of 1 ml of 15% trichloracetic acid. The tubes were centrifuged at 1100 × *g* for 10 min, and the inorganic phosphate hydrolyzed from reaction was measured in the supernatant by a colorimetric assay using KH_2PO_4 as standard (27). The ATPase activity assayed in the presence of 10 mM ouabain was subtracted from the total Mg^{2+}-dependent ATPase activity to calculate the activity of Na^+/K^+-ATPase.

Statistical analysis

All experiments were performed in triplicate. Correlation coefficients were calculated by linear regression analysis using the statistical program Microcal Origin 5.0 (OriginLab Corporation, Northampton, MA). Results are expressed as means ± SD. Statistical analyses were performed using the Student's *t* test for paired data. Differences were considered significant with $P < 0.05$.

TABLE 2. Na^+/K^+-ATPase, cytoplasmic Ca^{2+} concentration, and NO and peroxynitrite production in HAEC in the basal state (HAEC), after incubation with culture medium alone (control HAEC), with LDL from healthy subjects (C-LDL + HAEC), and with Hcy-LDL in the same healthy subjects (Hcy-LDL + HAEC)

	HAEC	Control HAEC	C-LDL + HAEC	Hcy-LDL + HAEC
Na^+/K^+-ATPase activity (μmol P_i·mg prot^{-1}·60 min^{-1})	0.98 ± 0.06	0.87 ± 0.15	1.01 ± 0.25	0.7 ± 0.01[a]
$[Ca^{2+}]_i$ (nM)	69 ± 3.37	73 ± 6.97	71 ± 16	142 ± 7.1[a]
NO production (nM/10^6 cell)	98 ± 7.0	97 ± 6.6	104 ± 22.0	75 ± 5.9[a]
Peroxynitrite production (nmol/mg protein)	0.74 ± 0.35	0.8 ± 0.3	0.9 ± 0.2	2.5 ± 0.2[a]

Medium ± SD is shown. P_i, Inorganic phosphate; prot, protein.
[a] $P < 0.001$ *vs.* HAEC, control HAEC and, C-LDL + HAEC.

Results

Na^+/K^+-ATPase activity was not significantly modified in HAEC incubated with C-LDL. A significant decrease was observed in cells incubated with Hcy-LDL compared with untreated HAEC (HAEC), HAEC incubated (control HAEC), and HAEC incubated with C-LDL (C-LDL HAEC; $P < 0.001$, HAEC *vs.* Hcy-LDL HAEC and C-LDL HAEC *vs.* Hcy-LDL HAEC; Table 2).

Hcy-LDL caused a significant modification in the endothelial cell cytoplasmic Ca^{2+} content compared with HAEC, control HAEC, and C-LDL HAEC ($P < 0.001$, Hcy-LDL HAEC *vs.* HAEC, control HAEC, and C-LDL HAEC; Table 2).

NO synthase activity was significantly decreased after incubation with Hcy-LDL ($P < 0.001$, Hcy-LDL HAEC *vs.* HAEC, control HAEC, and C-LDL HAEC; Table 2), whereas peroxynitrite production was markedly increased when the endothelial cells were incubated with Hcy-LDL compared with all the other conditions ($P < 0.001$, Hcy-LDL HAEC *vs.* HAEC, control HAEC, and C-LDL HAEC; Table 2).

A correlation between peroxynitrite levels and cytoplasmic Ca^{2+} content was found in HAEC incubated with Hcy-LDL ($n = 20$, $r = 0.976$; $P < 0.05$).

Discussion

Our previous study demonstrated that Hcy-LDL induced an increase in oxidative damage and a decrease of viability on human endothelial cells (11). Using the same experimental model, we demonstrated a significant decrease of Na^+/K^+-ATPase activity in HAEC incubated with Hcy-LDL with respect to HAEC incubated with C-LDL. Moreover, after incubation with Hcy-LDL, an increase of $[Ca^{2+}]_i$ was observed in HAEC.

The Na^+/K^+-ATPase is a plasma membrane enzyme responsible for maintaining the electrochemical gradient of sodium and potassium ions, and it plays an important role in the regulation of the ionic homeostasis in tissues and cells. Therefore, these results suggest that modifications of membrane ion exchanges at the cell surface could be involved in the damaging effect exerted by Hcy-LDL on HAEC. Modifications of the levels of intracellular calcium and release of Ca^{2+} from intracellular stores have been observed in endothelial cells incubated with lipoproteins (28).

Moreover, a significant increase in the peroxynitrite production that is directly correlated with a significant increase in $[Ca^{2+}]_i$ and a decrease in NO content probably due to its conversion into peroxynitrite by the inducible isoform (inducible NO synthase) that is Ca^{2+} independent have been observed in cells incubated with Hcy-LDL.

Previous studies have demonstrated that NO and its metabolites modulate Na^+/K^+-ATPase activity. In fact, Na^+/K^+-ATPase from different tissues has been observed to be inhibited by NO and peroxynitrite anion, even though the mechanism is still unknown. Using rat aorta, it has been demonstrated that the inhibition of Na^+/K^+-ATPase activity by high concentrations of L-arginine should be associated with activation of the endogenous synthesis of NO (29).

However, other hypotheses can be advanced to explain the modification of the activity of Na^+/K^+-ATPase in HAEC incubated with Hcy-LDL. Using rat brain, it has been demonstrated that Hcy significantly inhibits the enzyme activity. Considering the critical role exerted by Na^+/K^+-ATPase in brain, Streck *et al.* (30) proposed that the inhibition provoked by Hcy on the enzyme activity may be possibly related to the brain dysfunction characteristic of homocystinuria.

McCully (12) suggested that homocysteinylated LDLs are internalized by membrane receptors with intracellular release of Hcy by hydrolytic degradation of lipids through effect of cellular metabolism. Previous studies have demonstrated that Hcy induces endothelial cell dysfunctions by reducing intracellular NO concentrations and stimulating superoxide production (31). Therefore, we hypothesize that the decrease in NO content, the increase of peroxynitrite production and of $[Ca^{2+}]_i$ levels, and the inhibition of Na^+/K^+-ATPase activity observed in our experimental conditions in cultured HAEC after incubation with Hcy-LDL could be due to an effect exerted by Hcy released by internalized Hcy-LDL.

In conclusion, our results demonstrate that LDLs homocysteinylated *in vitro* induce alterations of enzyme activities and modulate NO metabolism of human endothelial cells with an increase in the levels of peroxynitrite. Previous studies have demonstrated that peroxynitrite exerts an oxidant effect because it initiates lipid peroxidation (31); therefore, we suggest that the increase in oxidative damage observed in our previous study (11) in HAEC incubated with homocysteinylated LDL could have involved alterations in NO metabolites.

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