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Effect of Homocysteinylation of Low Density Lipoproteins on Lipid Peroxidation of Human Endothelial Cells

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Abstract Homocysteine-thiolactone (HcyT) is a toxic product whose synthesis is directly proportional to plasma homocysteine (Hcy) levels. Previous studies demonstrated that the interaction between HcyT and low density lipoproteins (LDL) induces the formation of homocystamide-LDL adducts (Hcy-LDL). Structural and functional alterations of Hcy-LDL have been described and it has been suggested that homocysteinylation could increase atherogenicity of LDL. Oxidative damage of endothelial cells (EC) is considered to be a critical aspect of the atherosclerotic process. To further investigate the molecular mechanisms involved in the atherogenicity of homocysteinylated LDL, we studied the effect of interaction between Hcy-LDL and EC on cell oxidative damage, using human aortic endothelial cells (HAEC) as experimental model. Homocysteinylation of LDL was carried out by incubation of LDL, isolated from plasma of healthy normolipemic subjects, with HcyT (10–100 μM). In our experimental conditions, homocysteinylation treatment was not accompanied by oxidative damage of LDL. No modifications of apoprotein structure and physico-chemical properties were observed in Hcy-LDL with respect to control LDL (c-LDL), as evaluated using the intrinsic fluorescence of tryptophan and the probe Laurdan incorporated in lipoproteins. Our results demonstrated that Hcy-LDL incubated at 37°C for 3 h with HAEC, induced an oxidative damage on human EC with a significant increase of lipid hydroperoxides in cells incubated with Hcy-LDL with respect to cell incubated with c-LDL. The compositional changes were associated with a significant decrease viability in cells treated with Hcy-LDL. The relationship between the levels of –SH groups of LDL and the oxidative damage of HAEC has been demonstrated. These results suggest that Hcy-LDL exert a cytotoxic effect that is likely related to an increase in lipid peroxidation and oxidative damage of EC. J. Cell. Biochem. 92: 351–360, 2004.

Key words: low density lipoprotein; homocysteine-thiolactone; homocysteine; lipid peroxidation; endothelial cells; atherosclerosis

Several studies have demonstrated that an increase of the levels of homocysteine (Hcy) in human plasma represents an independent risk factor for cardiovascular disease and atherosclerosis [Anderson et al., 2000; Ueland et al., 2000]. The induction of the atherogenic process by hyperhomocysteinemia seems to be associated with an alteration of endothelial and smooth muscle cell functions [Harker et al., 1976; McDowell and Lang, 2000].

Jakubowski [1997] have demonstrated that Hcy-induced vascular damage could be due to homocysteine-thiolactone (HcyT), an Hcy reactive product formed in human cells from the enzymatic conversion of Hcy to the corresponding thioester. This hypothesis is supported by studies in vitro that have demonstrated that HcyT induces apoptosis and cell death in human endothelial cells (EC) [Mercie et al., 2000; Huang et al., 2001].

HcyT, under physiological concentrations of pH and temperature, readily reacts and acylates, by non-enzymatic nucleophilic addition, free amino groups, such as side-chain lysine residues of proteins (Hcy-proteins) [Jakubowski, 1999, 2000, 2002]. A good correlation between protein lysine content and their reactivity with HcyT

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has been established [Jakubowski, 1999]. Homocysteinylation results in incorporation of additional sulphydryl groups (−SH groups) into proteins with an increase in negative charge and in electrophoretic mobility, formation of intermolecular disulfide bonds with consequent formation of protein multimers in Hcy-proteins [Jakubowski, 1999]. These compositional and structural alterations are associated with an impairment of protein biological activities [Liu et al., 1997; Jakubowski, 1999].

Previous studies have demonstrated that plasma lipoproteins are susceptible to homocysteinylation [Vidal et al., 1986; Ferguson et al., 1999; Jakubowski, 1999; Ferretti et al., 2003a]. The interaction between HcyT and amino groups of apo-B lysyl residues of low density lipoproteins (LDL) induces the formation of homocystamide–LDL adducts (Hcy-LDL) [Vidal et al., 1986; Ferguson et al., 1999; Jakubowski, 1999]. Homocysteinylation of LDL is accompanied by an increase in density and in electrophoretic mobility [McCully, 1993; Naruszewicz et al., 1994]. The compositional and structural changes in Hcy-LDL are associated with functional alterations. Hcy-LDL are more susceptible to aggregation and to spontaneous precipitation. Moreover, using cultured macrophages, a higher uptake of Hcy-LDL by membrane receptor or by phagocitosis and a higher accumulation of intracellular cholesterol have been demonstrated [Naruszewicz et al., 1994]. Therefore, it has been suggested that homocysteinylation could increase the atherogenicity of LDL [McCully, 1993; Naruszewicz et al., 1994].

Alterations of functions and oxidative damages, induced by interactions with oxidized or glycated LDL on vascular endothelial cell, represent the early stages of the development of atherogenesis [Colaco and Roser, 1994; Khan et al., 1995; Pirillo et al., 2000; Salvayre et al., 2002].

The aim of this study was to further investigate the effect of homocysteinylation on apoprotein structure and physico-chemical properties of LDL isolated from human plasma using the analysis of the emission spectra of tryptophan intrinsic fluorescence and of the fluorescent probe Laurdan (6-dodecanoyl-2-dimethyl-aminonaphthalene). Moreover, we studied the effect of human LDL homocysteinylated in vitro on the oxidative damage and cell viability of EC using human aortic endothelial cells (HAEC) in culture as cell model.

MATERIALS AND METHODS

Materials

Endothelial cell growth medium (EGM-2), low serum growth supplement (LSGS), penicillin–streptomycin solution (10.000 U and 10 mg streptomycin/ml in 0.9% NaCl), L-glutamine (200 mM), ethylenediamine tetraacetic (EDTA), Trypsin–EDTA solution (10×), phosphate buffer solution (PBS), Trypan blue (0.4%), DL Hcy-thiolactone (DL-2-amino-4 mercaptobutyric acid 1,4-thiolactone) (HcyT), dithionitrobenzoic acid (DTNB), potassium bromide (KBr), methanol, reduced glutathione (GSH), xylenol orange [*o*-cresolsulfonephthalein-3′,3″-bis(methyliminodiacetic acid sodium salt)], ammonium iron (II) sulfate hexahydrate [$Fe(NH_4)_2(SO_4)_2$], sulphuric acid (H_2SO_4), butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Company (St. Louis, MO). Sephadex G-25 was obtained from Fluka AG (Buchs, Switzerland). Gel filtrations were performed on Econo-Pac chromatography columns from Bio-Rad (Hercules, CA). The fluorescent molecule Laurdan (6-dodecanoyl-2-dimethyl-aminonaphthalene) was obtained from Molecular Probes, Inc. (Eugene, OR).

Preparation of Human Plasma LDL

Blood was obtained by venipuncture from fasting, healthy normolipemic volunteers. Plasma was prepared by centrifugation at 3,000 rpm for 15 min and thereafter used for the preparation of LDL (1.025 and 1.063 g/ml) by single vertical spin gradient ultracentrifugation for 1 h and 30 min as described by Chung et al. [1986]. After dialysis at 4°C for 24 h against 10 mmol/L PBS (pH 7.4), protein concentration of LDL was determined by the method of Lowry et al. [1951]. LDL were sterilized on a 0.2 μm Millipore membrane before being incubated with HAEC.

Incubation of Human LDL With Hcy-Thiolactone

In a preliminary phase of the study, a pool of LDL isolated from healthy normolipemic subjects was used to investigate experimental conditions to homocysteinylate LDL (different times and temperature of incubation, increasing concentrations of HcyT).

An aliquot of LDL (100 μg of LDL protein) resuspended in 10 mmol/L PBS pH 8.2 was incubated at 37°C in the absence (c-LDL) or in the presence of Hcy-thiolactone (10–100 μmol/

L) (Hcy-LDL) for different times (0–3 h). The mixture was incubated with gentle stirring for the indicated times and passed through a Sephadex G-25 column equilibrated with 10 mmol/L PBS, pH 8.2, in order to separate the unreacted Hcy-thiolactone.

The reaction of homocysteinylation of LDL was verified by the study of the increase in –SH groups in Hcy-HDL with respect to control LDL [Vidal et al., 1986; Ferguson et al., 1999].

The levels of sulphydryl groups were assayed using the DTNB reagent [Hu, 1994]. An aliquot of LDL (100 μg) treated in different experimental conditions, was incubated with 0.25 mol/L Tris-HCl pH 8.2 and 20 mmol/L EDTA in the presence of 0.1 mmol/L DTNB and absolute methanol. After incubation for 20 min at room temperature, LDL were centrifuged at 3,000 rpm for 10 min. The absorbance of the supernatant was measured at 412 nm. The levels of –SH groups were quantified using a stock solution of 1 mmol/L glutathione reduced (GSH) [Hu, 1994]. The concentrations of –SH groups are given in terms of nanomoles per milligram of LDL protein.

Cell Culture

HAEC (a gift of Prof. L. Mazzanti-Ancona, Italy) were grown in EGM-2, containing 1% L-glutamine (200 mmol/L) and 1% penicillin/streptomycin and supplemented with LSGS at 37°C in a humidified atmosphere of 5% CO_2 [Freshney, 2000]. After 2 or 3 days, cells reached 90–95% confluence as observed by a light microscope (10×) and were used for the experiments

Incubation of HAEC With Low Density Lipoprotein (LDL)

HAEC were incubated for 3 h in EGM-2, (at 37°C and 5% CO_2) in the absence (control HAEC) or in the presence of control or homocysteinylated LDL at a concentration of 100 μg/ml (Hcy-treated-LDL) [Rabini et al., 2002]. At the end of incubation with c-LDL or Hcy-treated-LDL, the monolayers were removed by scraping and resuspended in 10 mmol/L PBS, pH 7.4 for evaluation of the levels of lipid hydroperoxides and cell viability.

Evaluation of Lipid Peroxidation in HAEC and in LDL

The extent of lipid peroxidation in HAEC and LDL treated in different experimental conditions, was evaluated by measuring the levels of hydroperoxides using the ferrous oxidation xylenol orange assay (FOX-assay) in agreement with our previous studies [Ferretti et al., 2003b,c]. Briefly, the levels of hydroperoxides in cells were evaluated by adding 1,350 μl of FOX-reagent (100 μmol/L xylenol orange, 250 μmol/L Fe^{++}, 25 mM H_2SO_4, and 4 mmol/L BHT in 90% methanol (v/v)) to HAEC previously pelletted and resuspended in 650 μl of 10 mM PBS. After 20 min of incubation at 37°C, cells were centrifuged at 4,500 rpm for 15 min and the absorbance of supernatant was evaluated at 560 nm.

The levels of hydroperoxides of c-LDL and Hcy-LDL were determined by adding 1,350 μl of FOX-reagent to 650 μl of sample following the experimental procedure previously described for cells [Jiang et al., 1992]. T-butyl hydroperoxide solution was used as standard. The results are presented as nmol of hydroperoxides for 10^6 cells or as nmol of hydroperoxides for 100 μg of lipoproteins.

Fluorescence Studies

The intrinsic fluorescence of tryptophan in c-LDL and in Hcy-LDL was studied at 37°C using 100 μg LDL protein resuspended in 10 mmol/L PBS (pH 7.4) (final volume, 2 ml). Emission spectra were recorded using Perkin-Elmer LS 50B spectrofluorimeter using 280 nm as excitation wavelength [Dousset et al., 1994].

The incorporation of the fluorescent probe Laurdan (2-dodecanoyl-2-dimethyl-aminonaphthalene) was carried out incubating 100 μg of c-LDL or Hcy-LDL protein with 2 μl of a probe solution (final concentration, 1 μmol/L) at room temperature [Dousset et al., 1994; Ferretti et al., 2002]. The emission spectra were obtained using an excitation wavelength of 340 nm. The generalised polarization value (Gp) of Laurdan was calculated as previously described by Parasassi et al. [1991]: $Gp = (I_{435} - I_{490})/(I_{435} + I_{490})$, where I_{435} and I_{490} are the intensities at the blue and red edges of the emission spectrum, respectively, and correspond to the fluorescent emission maxima in the gel and in the liquid-crystalline phases of a phospholipid bilayer. The position of the maximum emission and Gp value of Laurdan are sensitive to the polarity and to the molecular order of the microenvironment surrounding the probe [Parasassi et al., 1991].

Cell Viability Assay

The viability of HAEC was evaluated using Trypan Blue assay [Freshney, 2000]. In fact, the loss of membrane integrity in dead and dying cells allows the preferential uptake of labels like Trypan Blue. Briefly, cells were pelleted and resuspended in 5 mmol/L PBS, 10 μl of the cell suspension were mixed with 25 μl of 0.4% Trypan Blue and 15 μl of 5mmol/L PBS (pH 7.4). After 5 min of incubation at room temperature, cell suspension was transferred to the hemocytometer chamber and counted using a light microscope (10×), the blue stained cells were considered non-viable. The results were expressed as cell viability percentage.

Statistics

All experiments were performed in duplicate and were repeated for almost three times. Results were presented as the mean ± SD. The difference of the results obtained in different experimental conditions was analyzed using Student's t test. Values were considered to be significant at P values less then 0.05. Correlation coefficients were calculated by linear regression analysis using the statistical program Microcal Origin 5.0 (OriginLab, Northampton, MA).

RESULTS

Effect of HcyT on Human LDL Lipid Peroxidation

The mean level of –SH groups in untreated LDL was 19.8 ± 5.3 nmol/mg of proteins. A time dependent increase of –SH groups was observed in LDL incubated with HcyT (10–100 μmol/L) (Hcy-LDL) with respect to LDL incubated alone (c-LDL) (Fig. 1).

The increase in –SH groups in Hcy-LDL was significant using 100 μmol/L HcyT. After 2 h of incubation a two fold increase in –SH groups with respect to c-LDL has been observed ($P <$ 0.001 vs. c-LDL) (Fig. 2). Therefore, all the following experiments were carried out using LDL incubated in these experimental conditions (incubation with 100 μmol/L for 2 h). The increase in the levels of –SH groups confirms the reaction between HcyT and apo-B lysyl residues of LDL, in agreement with previous studies [Vidal et al., 1986; Ferguson et al., 1999; Jakubowski, 1999].

The level of lipid hydroperoxides in untreated LDL was 0.58 ± 0.20 nmol/100 μg. A slight increase was observed after 2 h of incubation both in LDL incubated alone and in LDL incubated with 100 μmol/L HcyT. The differences were not significative (0.68 ± 0.19 nmol/100 μg and 0.72 ± 0.16 in c-LDL and Hcy-LDL, respectively) (Fig. 2). These results suggest that homocysteinylation treatment of LDL is not associated with oxidative damage in our experimental conditions.

Effect of HcyT on Tryptophan and Laurdan Fluorescence Properties of LDL

To investigate whether homocysteinylation induces modifications of apoprotein conformation and physico-chemical properties of LDL, we

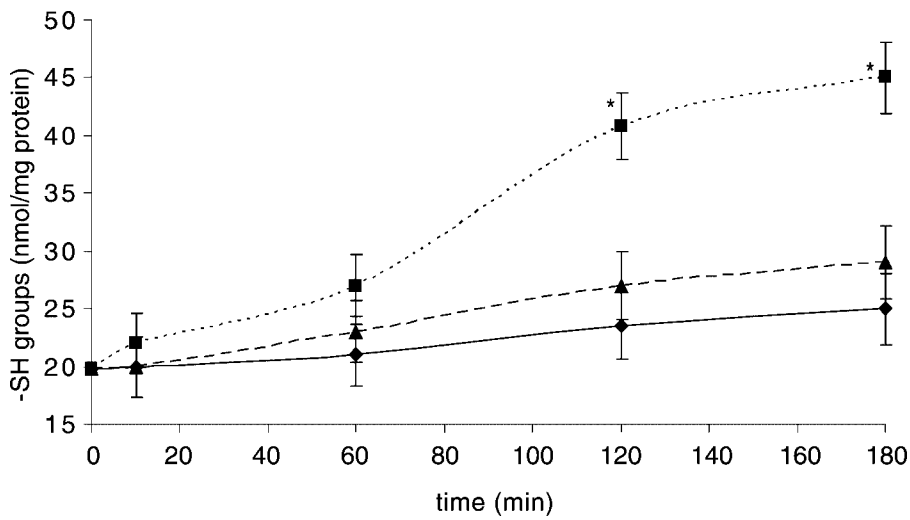

Fig. 1. Time dependent increase in the levels of –SH groups in LDL incubated in the absence (c-LDL) (–◆–) and in the presence of different concentrations of HcyT (Hcy-LDL) (–▲– 10 μM and –■– 100 μM HcyT). *$P <$ 0.001 vs. levels of –SH groups in c-LDL.

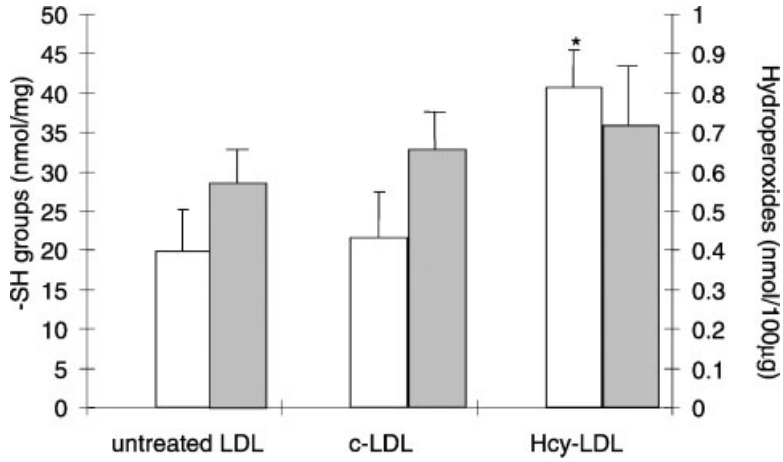

Fig. 2. Levels of –SH groups (□) and levels of lipid hydroperoxides (■) in untreated and in LDL incubated for 2 h in the absence (c-LDL) or in the presence of 100 μM HcyT (Hcy-LDL). *$P < 0.001$ vs. levels of hydroperoxides in untreated cells.

compared the emission spectra of tryptophan (Trp) and of the fluorescent probe Laurdan, in control and Hcy-LDL.

In c-LDL the emission maximum of Trp emission spectra was 340.2 ± 0.9 nm, in agreement with previous studies [Dousset et al., 1994; Parasassi et al., 2001] and was not significantly modified in Hcy-LDL (Table I).

The position of the fluorescent emission maximum and the Gp value of Laurdan incorporated in control LDL were 422.7 ± 1.1 nm and 0.559 ± 0.007, respectively, as previously observed [Dousset et al., 1994; Ferretti et al., 2002]. As summarized in Table I, the emission maximum and Gp value were not significantly modified in Hcy-LDL, suggesting that the physico-chemical properties of LDL are not modified by homocysteinylation treatment.

TABLE I. Emission Maximum of Trp Fluorescence (a) and Gp Value and Emission Maximum of Laurdan Fluorescence (b) of Untreated LDL and of LDL Incubated in the Absence (c-LDL) or in the Presence of 100 μM Hcy-Thiolactone (Hcy-LDL)

(a) Trp fluorescence		
	λ (nm)	
Untreated-LDL	339.7 ± 1.1	
c-LDL	340.2 ± 0.9	
Hcy-LDL	340.9 ± 1.0	
(b) Laurdan fluorescence		
	Gp	λ (nm)
Untreated-LDL	0.558 ± 0.009	422.3 ± 1.0
c-LDL	0.559 ± 0.007	422.7 ± 1.1
Hcy-LDL	0.565 ± 0.008	423.1 ± 0.9

Effect of LDL and Hcy-LDL Lipid Peroxidation of HAEC

To investigate the effect of homocysteinylated LDL on oxidative damage of EC, we evaluated the levels of lipid hydroperoxides and cell viability in HAEC incubated in the presence of c-LDL or Hcy-LDL.

The level of lipid hydroperoxides in untreated HAEC was 0.91 ± 0.59 nmol/10^6 cells and was not significantly modified during the incubation at 37°C for 3 h in the absence of lipoproteins (0.96 ± 0.40 nmol/10^6 cells, ns). Moreover, the cell viability was not significantly modified with respect to untreated cells (Fig. 3).

A slight, but not significant, increase in the levels of hydroperoxides was observed in cells incubated in the presence of c-LDL with respect to cells incubated alone (1.28 ± 0.74 nmol/10^6 cell) (Fig. 3). Higher levels of hydroperoxides have been observed in HAEC incubated with Hcy-LDL with respect to c-LDL-treated cells (8.08 ± 0.96 nmol/10^6 cell, $P < 0.001$). These results demonstrated that Hcy-LDL induce oxidative damage on EC.

Cell viability was not significantly modified in HAEC incubated with c-LDL with respect to control cells (88.0 ± 10 and $81.2 \pm 9\%$ in control and c-LDL treated cells). A significant decrease in cell viability has been observed in cells

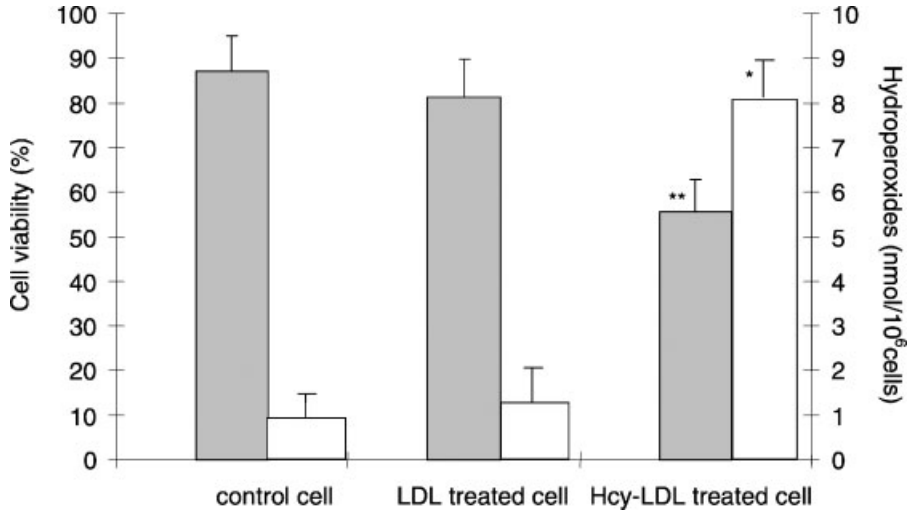

Fig. 3. Levels of hydroperoxides (□) and cell viability (■) in HAEC incubated in the absence (control cell) or in the presence of c-LDL (LDL-treated cell) or Hcy-LDL (Hcy-LDL-treated cell).*$P < 0.001$ vs. levels of hydroperoxides in control cells; **$P < 0.001$ vs. cell viability in control cells.

treated with Hcy-LDL ($55.4 \pm 8\%$) ($P < 0.001$ vs. c-LDL treated cells) (Fig. 3).

Correlations

Using linear regression analysis we studied the correlations between the levels of –SH groups in control and homocysteinylated LDL and the levels of lipid hydroperoxides and the viability in HAEC treated in different experimental conditions. A significant positive correlation was established between the levels of –SH groups associated to control and Hcy-LDL and the levels of hydroperoxides of cells ($r = 0.96$; $P < 0.001$, $n = 10$) (Fig. 4a). A negative significant correlation between the levels of –SH groups associated to lipoproteins and cell viability was observed ($r = -0.97$; $P < 0.001$, $n = 10$) (Fig. 4b).

These results demonstrated a relationship between the compositional changes induced by homocysteinylation on LDL and the increase of oxidative damage and the decrease of cell viability. Moreover, in our experimental conditions, a significant negative correlation between cell viability and the extent of oxidative damage of cells was demonstrated ($r = -0.98$; $P < 0.001$, $n = 10$), suggesting that the compositional changes in cells treated with Hcy-LDL reflect in a decrease in cell viability in agreement with previous studies in cells oxidized in different experimental conditions [Ferretti et al., 2003b,c].

DISCUSSION

Several studies have demonstrated that HcyT induces apoptosis and cell death in human (EC) [Mercie et al., 2000; Huang et al., 2001], therefore, it has been suggested that Hcy-induced vascular damage could be due to HcyT [Jakubowski, 1997, 2000].

Recent studies have suggested that the toxicity of Hcy-thiolactone could be related to its ability to homocysteinylate proteins, in fact alterations of structural and functional properties were observed in Hcy-proteins [Liu et al., 1997; Jakubowski, 1999].

Homocysteinylation of lipoproteins can be induced in vitro by incubation with Hcy-thiolactone; therefore lipoproteins incubated with Hcy-thiolactone represent an useful experimental model to study the structural and functional alterations of homocysteinylated lipoproteins [Vidal et al., 1986; Ferguson et al., 1999; Jakubowski, 1999; Ferretti et al., 2003a].

The significant increase in the levels of –SH groups in LDL incubated with Hcy-thiolactone, demonstrates that the reaction between HcyT and apo-B lysyl residues of LDL occurs in our experimental conditions and confirms that LDL are sensitive to homocysteinylation treatment [Vidal et al., 1986; Ferguson et al., 1999; Jakubowski, 1999]. Assuming a molecular weight of 500 kDa of apo-B and about 200 lysyl residues per apo-B molecule [Ferguson et al.,

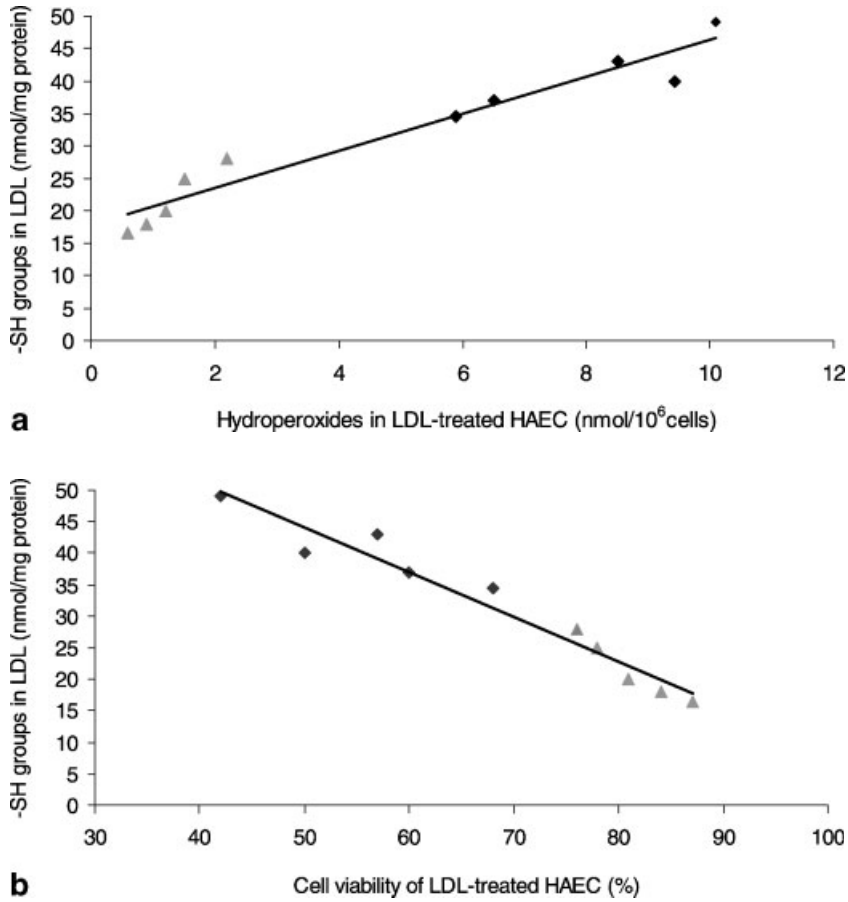

Fig. 4. **a**: Correlation between the levels of –SH groups in low density lipoproteins and lipid hydroperoxides in HAEC treated with lipoproteins (control LDL, △ Hcy-LDL ◆) ($r = 0.96$; $P < 0.001$, $n = 10$). **b**: Correlation between the levels of –SH groups in low density lipoproteins and cell viability of HAEC treated with lipoproteins (control LDL, △ Hcy-LDL ◆) ($r = -0.97$; $P < 0.001$, $n = 10$).

1999], we estimate that about 10% of groups of lysine were acylated in LDL incubated for 2 h in the presence of 100 μM HcyT.

In our experimental conditions, homocysteinylation treatment was not associated with oxidative damage of LDL as demonstrated by similar levels of lipid hydroperoxides in Hcy-LDL and c-LDL, in agreement with previous studies [McCully, 1993; Naruszewicz et al., 1994; Ferguson et al., 1999]. Moreover, studies in vivo have shown that LDL isolated from hyperhomocysteinaemic patients are not more susceptible to oxidation with respect to LDL isolated from plasma of healthy subjects [Blom et al., 1995].

The analysis of Trp fluorescence spectra has not shown significant changes in the position of the maximum emission of Trp in Hcy-LDL. Moreover, using the fluorescent probe Laurdan incorporated in Hcy-LDL, no modifications of the Gp value and of position of the emission maximum of the probe have been observed. These results demonstrated that apoprotein structure and physico-chemical properties are not modified in LDL homocysteinylated in our experimental conditions.

Previous studies have demonstrated that Hcy-LDL became more aggregated and were more susceptible to spontaneous precipitation with respect to control LDL [Naruszewicz et al., 1994]. Moreover, using cultured human monocyte-derived macrophages, Naruszewicz et al. [1994] have shown alterations of the interactions between homocysteinylated-LDL and cells with an higher internalisation of Hcy-LDL by membrane receptors or by phagocytosis and higher intracellular cholesterol accumulation. Therefore, it has been hypothesised that homocysteinylation represents an atherogenic modification of LDL.

The effect of Hcy-lipoproteins on EC has not been previously studied. In the present study we demonstrated, for the first time, an increase in the levels of hydroperoxides in HAEC incubated in the presence of Hcy-LDL with respect to cells treated with control LDL. These results suggest that the interaction between Hcy-LDL and cells induced an oxidative damage in HAEC. The compositional changes in HAEC incubated with Hcy-LDL were associated with a significant decrease in cell viability and a significant positive correlation has been established between the levels of lipid hydroperoxides in cells and the decrease of cell viability. These results confirm that lipid peroxidation induces endothelial cell injury and death in agreement with previous studies in cells oxidized by different agents [Hennig and Chow, 1988; Ferretti et al., 2003b,c].

In our experimental conditions, a positive significant correlation has been established between the levels of –SH groups in Hcy-LDL and c-LDL and the levels of lipid hydroperoxides in cells incubated with lipoproteins. Therefore, we hypothesise that the citotoxicity of Hcy-LDL could be related to the compositional changes of Hcy-LDL and to the increase in –SH groups at the surface of Hcy-LDL with respect to c-LDL.

Some hypotheses can be advanced to explain the oxidative damage triggered by Hcy-LDL on HAEC cells. Homocysteinylated LDL are internalized by membrane receptors with intracellular release of Hcy by hydrolytic degradation. Hcy presumably could lead to oxidative degradation of lipids through effect of cellular metabolism [McCully, 1993]. The ability of thiols (such as D,L Hcy, L-cysteine) to generate partially reduced oxygen species such as O_2^-, H_2O_2, and OH^- and to initiate lipid peroxidation has been widely demonstrated [Rowley and Halliwell, 1982; McDowell and Lang, 2000]. Hcy, even at low concentrations 0.050–0.10 mmol/L, is cytotoxic and induces cell injury and oxidative damage in cultured EC with generation of reactive oxidative species (ROS), formation of superoxide and hydrogen peroxide [Starkebaum et al., 1986; Outinen et al., 1998; McDowell and Lang, 2000; Drunat et al., 2001] and increase in the levels in lipid peroxidation products [Gao and Xue, 2003].

As far as concerns the physiological relevance of our results, Jakubowski et al. [2000] have shown that protein homocysteinylation realises in vivo. LDL exhibit a lower reactivity toward Hcy-thiolactone with respect to other plasma proteins (albumin, γ-globulin), however, Hcy-lipoproteins (Hcy-LDL and Hcy-HDL) have been determined also in plasma of healthy subjects [Jakubowski et al., 2000; Jakubowski, 2002] and it has been demonstrated that protein homocysteinylation is related to plasma Hcy levels [Jakubowski et al., 2000; Jakubowski, 2002]. Congenital or acquired homocysteinemia has been observed in patients affected by pathological conditions such as vascular diseases [Anderson et al., 2000; Ueland et al., 2000], neurological diseases [Clarke et al., 1998], renal failure [Suliman et al., 2001], type 1 diabetes [Agullo-Ortuno et al., 2002], cancer [Wu and Wu, 2002], therefore higher levels of HcyT and of homocysteinylated lipoproteins might be expected in these diseases.

Vascular EC play an active role in the physiological processes of vessel tone regulation and vascular permeability and form a border separating deeper layers of the blood vessel wall and cellular interstitial space from the blood and circulating cells. Therefore, oxidative damage to EC induced by LDL play an important role in the development of atherosclerosis.

Chemical and biological modifications of lipoproteins, particularly LDL, are believed to increase their atherogenicity. In fact covalent alterations of LDL by acetylation, oxidation or glycation, increase lipoprotein uptake and cholesterol deposition within cultured cells. Moreover oxidized or glycated LDL induce lipid peroxidation in cultured cells [Colaco and Roser, 1994; Pirillo et al., 2000; Salvayre et al., 2002].

The metabolic relationship between Hcy and Hcy-thiolactone and the molecular mechanism by which these molecules are involved in the development of atherosclerosis and other human diseases deserve further investigation.

Our results demonstrated, for the first time, that LDL homocysteinylated in vitro are able to induce oxidative damage and death on human EC and support the hypothesis advanced by previous studies [McCully, 1993; Naruszewicz et al., 1994] that the compositional changes induced by homocysteinylation on LDL could increase the atherogenicity of LDL.

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