Effect of Homocysteinylation on Human High-Density Lipoproteins: A Correlation With Paraoxonase Activity

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We investigated the effect of homocysteine (Hcy)-thiolactone on the activity of the enzyme paraoxonase (PON) associated with human high-density lipoprotein (HDL-PON). HDL were isolated from plasma of normolipidemic subjects. The increase in the levels of sulfhydryl groups (−SH) in HDL incubated with Hcy-thiolactone demonstrates that homocysteinylation of HDL occurs. The increase of −SH groups correlated with the basal values of HDL-PON activity ($r = -0.73$, $P < .001$, and $r = -0.70$, $P < .002$ using 10 μmol/L and 1 mmol/L Hcy-thiolactone, respectively) suggesting a relationship between the susceptibility of HDL to homocysteinylation and HDL-PON activity. A decrease in the activity of the enzyme HDL-PON was observed in homocysteinylated HDL (Hcy-HDL). The negative correlation established between the basal levels of HDL-PON activity and the percentage decrease of HDL-PON activity ($r = -0.76$, $P < .001$, and $r = -0.86$, $P < .001$ using 10 μmol/L or 1 mmol/L Hcy-thiolactone, respectively) suggests that subjects with higher HDL-PON activity have a lower decrease in PON activity with respect to subjects with lower HDL-PON activity. The positive correlation established between the percentage decrease of PON activity and the percentage increase of –SH groups in Hcy-HDL ($r = 0.80$, $P < .001$, and $r = 0.76$, $P < .001$ in HDL incubated in the presence of 10 μmol/L and 1 mmol/L Hcy-thiolactone, respectively) suggests that the modifications of HDL-PON activity are likely related to the compositional changes at the lipoprotein surface of Hcy-HDL. The enzyme PON contributes to the protective role of HDL against the oxidative damage and against toxicity exerted by Hcy involved in the development of atherosclerosis. Therefore the significant decrease of the enzyme activity in HDL incubated with Hcy-thiolactone suggests that homocysteinylation could render HDL less protective against oxidative damage and against toxicity of Hcy-thiolactone.

THERE IS GROWING evidence that elevated plasma levels of homocysteine (Hcy) are associated with an increased risk of cardiovascular disease[1] and atherosclerosis.[2] The pathogenic role of Hcy was related to its ability to generate superoxide ion and hydrogen peroxide, which promote low-density lipoprotein (LDL) lipid peroxidation[3] and damage to arterial endothelium.[4] Moreover, Hcy modifies coagulation factor levels so as to encourage clot formation and enhances the proliferation of smooth muscle cell in the arterial wall.[5]

Recent studies hypothesized that the Hcy-induced vascular damage could be due to Hcy-thiolactone, an Hcy-reactive product formed in several cell types as a result of editing reactions of some aminoacyl-tRNA synthetase.[6,7] The synthesis of Hcy-thiolactone is directly proportional to plasma Hcy/methionine ratio.[7] Chronic infusions of Hcy-thiolactone in experimental animals, such as rabbits[2] and baboons,[8] cause atherosclerosis and it was demonstrated that high doses of Hcy-thiolactone cause elevation of plasma cholesterol, LDL, and very-low-density lipoprotein (VLDL) that represents an atherogenic risk.[9] Moreover, it was demonstrated that high concentrations of Hcy-thiolactone could induce apoptosis of cultured vascular endothelial cells.[10] However, the molecular mechanisms by which Hcy-thiolactone could exert its role in Hcy-induced vascular damage are not still completely understood.

It was proposed that Hcy-thiolactone could react with lysine residues of proteins (protein-N-Hcy) and damage protein structure.[11,12] The interaction between Hcy-thiolactone and LDL causes LDL aggregation, and a higher uptake of homocysteinylated LDL (Hcy-LDL) by cultured macrophages was demonstrated.[2]

The existence of thiolactonase (Htase), a high-density lipoprotein (HDL)-associated enzyme able to hydrolyze Hcy-thiolactone to Hcy, was recently demonstrated by Jakubowski et al.[13] This protective enzymatic mechanism against Hcy-thiolactone toxicity is identical with paraoxonase (PON), which plays a protective role against lipid peroxidation of LDL[14] and HDL[15] in human plasma. The enzymatic activity of PON varies widely among healthy humans. The two common polymorphism of PON are M or L at positions 55 (methionine or leucine, respectively) and Q or R at position 192 (glutamine or arginine, respectively).[16,17] The R and Q isoforms are associated with high and low PON activity with paraoxon and linked to coronary artery disease (CAD) risk.[17,18] Recently, Jakubowski et al[19] reported that the L55 and R192 alleles are associated with high serum Htase activity. Moreover, it was demonstrated that high serum Htase activity affords better protection against serum protein homocysteinylation with respect to low serum Htase activity.[19]

The effect of Hcy-thiolactone on human HDL has not been investigated until now. The aim of the present study was to investigate the relationship between HDL-PON activity in normolipemic subjects and the susceptibility of HDL to homocysteinylation and to study the effect of homocysteinylation on HDL-PON activity.

MATERIALS AND METHODS

Materials

Hcy-thiolactone, dithionitrobenzoic acid (DTNB), diethyl *p*-nitrophenyl phosphate (paraoxon), potassium bromide (KBr) trichloro-acetic acid (TCA), 2-thiobarbituric acid (TBA), and phosphate-buffered saline (PBS) were provided by Sigma Chemical (St Louis, MO). Sephadex G-25 was obtained from Fluka AG (Buchs, Switzerland). Gel

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Submitted January 28, 2002; accepted September 3, 2002.

Supported by a grant from MURST (60% G.C. and G.F.).

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0026-0495/03/5202-0014$35.00/0

doi:10.1053/meta.2003.50033

filtrations were performed on Econo-Pac chromatography columns from Bio-Rad (Hercules, CA).

Preparation of Human HDL

Blood was obtained by venipuncture from fasting 16 male healthy donors. Mean age was 30 ± 4 years and mean body mass index (BMI) was 23 ± 3 kg/m^2. All subjects were normolipidemic as demonstrated by the levels of plasma lipids (total cholesterol [TC], 205 ± 10 mg/dL), triglycerides (TG, 80 ± 6 mg/dL), and HDL cholesterol (HDL-C, 40 ± 8 mg/dL). The mean level of glycemia was 80 ± 11 mg/dL. Smokers were excluded from the study as previous studies demonstrated alterations of paraoxonase activity in smokers.[20]

The study was approved by the local ethics committee and informed consent was obtained from each subject before the investigation.

Blood was collected in heparin-containing vacutainer tubes. Plasma was prepared by centrifugation at 3,000 rpm for 20 minutes and thereafter used for the preparation of lipoproteins. HDL (d = 1.063 to 1.120 g/mL) were isolated by single vertical spin density gradient ultracentrifugation for 1.30 hours at 65,000 rpm[21] and dialyzed at 4°C for 24 hours against PBS. Lipoproteins have been used within 24 hours after isolation.

Incubation of Human HDL With Hcy-Thiolactone

In this study the homocysteinylation of HDL was performed following the experimental conditions described previously for LDL.[22] In a preliminary phase of the study, a pool of HDL isolated from healthy normolipemic subjects was used to investigate the effect of homocysteinylation in different experimental conditions (different concentrations of Hcy-thiolactone, different times of incubation [10 to 180 minutes]).

An aliquot of HDL (100 μg of HDL protein) resuspended in 100 mmol/L PBS pH 8.2 was incubated at 25°C in the absence or in the presence of Hcy-thiolactone (10 μmol/L to 1 mmol/L) according to Vidal et al.[22] After different times of incubation, the mixture was passed through a Sephadex G-25 column equilibrated with 100 mmol/L PBS, pH 8.2, in order to separate the unreacted Hcy-thiolactone.

Evaluation of Homocysteinylation of HDL

The reaction of homocysteinylation of HDL was verified by the study of the increase in −SH groups in HDL incubated at 25°C in the presence of increasing concentrations of Hcy-thiolactone (Hcy-HDL) with respect to HDL incubated alone (basal HDL).[22]

The levels of sulfhydryl groups were assayed using the DTNB reagent.[23] An aliquot of basal or treated HDL (100 μg) 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 minutes at room temperature, HDL were centrifuged at 3,000 × g for 10 minutes. The absorbance of supernatant was measured at 412 nm. The levels of SH groups were quantified using a stock solution of 1 mmol/L glutathione reduced (GSH).[23] The concentrations of SH groups are given in terms of nanomoles per milligram HDL protein.

HDL-PON Activity Assay

PON activity was assayed in plasma and in HDL using 100 μg of HDL resuspended in 5 mmol/L Tris-HCl pH 7.4 containing 0.15 mol/L NaCl, 4 mmol/L $MgCl_2$, and 2 mmol/L $CaCl_2$. The reaction was initiated by adding 1 mmol/L paraoxon (dietyl-*p*-nitrophenyl phosphate) and the increase in the absorbance was monitored at 410 nm. The amount of 4-nitrophenol formed was calculated from the molar extinction coefficient 1,310 mol/L^{-1} · cm^{-1}. One unit of PON activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions.[24]

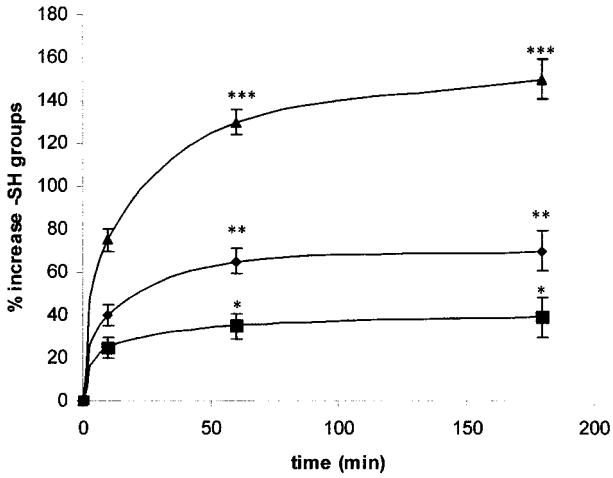

Fig 1. Time-dependent increase of −SH groups in HDL incubated in the presence of different concentrations of Hcy-thiolactone (■, 10 μmol/L; ◆, 100 μmol/L; and ▲, 1 mmol/L Hcy-thiolactone). Results are presented as percentage increase with respect to control HDL. (*P < .05; **P < .003;*P < .002 *v* HDL incubated for 10 minutes in the presence of 10 μmol/L, 100 μmol/L, and 1 mmol/L Hcy-thiolactone, respectively).**

Analytical Methods

Protein concentration of control HDL was determined by the method of Lowry et al.[25] The extent of lipid peroxidation of control and Hcy-HDL was evaluated by measuring the thiobarbituric acid–reactive substances (TBARS) as described by Buege and Aust.[26]

Statistics

All experiments were performed in triplicate. Correlation coefficients were calculated by linear regression analysis using the statistical program Microcal Origin 5.0 (OriginLab, Northampton, MA). Student's t test was used to analyze the significance of results obtained in untreated and treated HDL. Values were considered to be significant at P less then .05.

RESULTS

Effect of Hcy-Thiolactone on HDL

The mean level of −SH groups in basal HDL was 1.88 ± 0.86 nmol/mg of proteins. As shown in Fig 1, significant changes of the levels of −SH groups were observed, even after a brief time of incubation (10 minutes) in HDL incubated with Hcy-thiolactone (10 μmol/L to 1 mmol/L) with respect to control HDL. The increase in −SH groups was dependent on the concentration of Hcy-thiolactone.

The increase in −SH groups suggests that in our experimental conditions, homocysteinylation of $-NH_2$ groups of HDL occurs, which is in good agreement with previous studies in LDL.[22]

The levels of TBARS were not significantly modified in HDL incubated with Hcy-thiolactone (Hcy-HDL) with respect to basal HDL (the levels of TBARS were 1.1 ± 8 nmol/mg in untreated HDL and 1.07 ± 0.9 nmol/mg in Hcy-HDL), suggesting that oxidative damage does not occur during incubation of HDL with Hcy-thiolactone.

Correlation Between Homocysteinylation of HDL and HDL-Paraoxonase Activity

The effect of homocysteinylation was studied in HDL of 16 subjects with values of PON activity in plasma ranging between 1,120 U/mL and 6,100 U/mL. The activity of PON in HDL isolated from plasma of the same subjects ranged between 79.3 U/mg to 781 U/mg (median, 178.5 U/mg). As shown in Fig 2 a correlation was established between PON activity in plasma and in HDL, which is in agreement with localization of plasma PON at the surface of HDL.[16]

To investigate whether the susceptibility of HDL to homocysteinylation correlates with PON activity associated with HDL, subjects were divided into 2 subgroups with HDL-PON activity below or above the median value (178.5 U/mg), respectively. The mean values of −SH groups in HDL of subjects with HDL-PON activity lower than the median value (L-HDL-PON), were slightly but significantly higher with respect to HDL of subjects with higher activity (H-HDL-PON) (2.5 ± 0.62 *v* 1.2 ± 0.53 nmol/mg of HDL proteins in L-HDL-PON and H-HDL-PON, respectively; $P < .001$).

The comparison of the effect of homocysteinylation in the 2 groups of HDL showed that the percentage increase of −SH groups was higher in subjects with lower HDL-PON activity with respect to subjects with higher HDL-PON either using 10 μmol/L or 1 mmol/L Hcy-thiolactone. The differences were statistically significant (Fig 3). These results suggest that the susceptibility of HDL to homocysteinylation, as evaluated by determination of the increase of −SH groups, is related to HDL-PON activity and that HDL with lower PON activity are more susceptible to homocysteinylation with respect to HDL with higher activity. The hypothesis is supported by the negative correlation established between the basal values of HDL-PON activity and the percentage increase of −SH groups in the whole group of subjects ($r = -0.73$, $P < .001$, and $r = -0.70$, $P < .002$ using 10 μmol/L and 1 mmol/L Hcy-thiolactone, respectively) (Table 1).

Effect of Homocysteinylation on HDL-PON Activity

To investigate whether homocysteinylation of HDL reflects in modifications of the enzyme activity of HDL-PON, the activity of paraoxonase was studied in Hcy-HDL with respect to untreated HDL. As shown in Fig 4, HDL-PON activity was lower in Hcy-HDL with respect to untreated HDL. However, the decrease was realized at a different extent in HDL of the whole group of subjects.

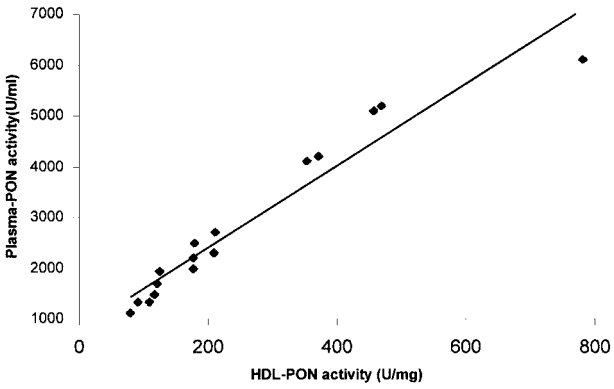

Fig 2. Correlation between the activity of paraoxonase in plasma (Plasma-PON activity) and in HDL (HDL-PON activity) ($r = 0.96$; $P < .001$, n = 16).

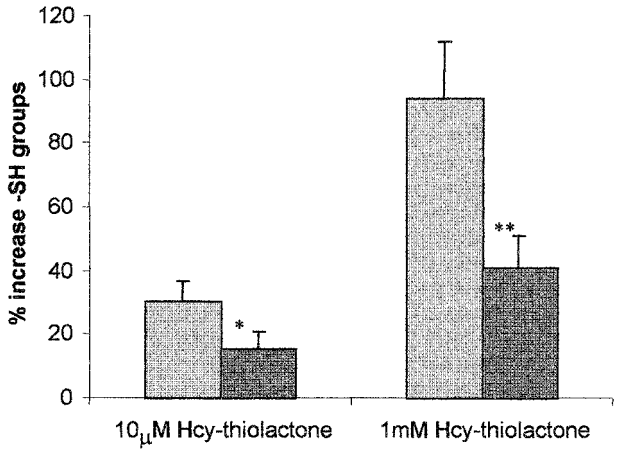

Fig 3. Percentage increase of −SH groups in HDL after incubation with 10 μmol/L and 1 mmol/L Hcy-thiolactone. Subjects with HDL-PON activity below (■) or above (■) the median value (178.5U/mg). Results are presented as percentage increase with respect to control HDL. *$P < .001$ *v* HDL with PON activity below the median value; **$P < .001$ *v* HDL with PON activity below the median value.

The comparison of the effect of homocysteinylation on HDL-PON in the 2 subgroups of subjects (L-HDL-PON and H-HDL-PON) showed that the percentage decrease of HDL-PON activity was lower in subjects with H-HDL-PON than in subjects with L-HDL-PON either using 10 μmol/L or 1 mmol/L Hcy-thiolactone. The differences were statistically significant (Fig 5).

The negative correlation established between the basal levels of HDL-PON activity and the percentage decrease of HDL-PON activity in Hcy-HDL ($r = -0.76$, $P < .001$, and $r = -0.86$, $P < .001$ using 10 μmol/L or 1 mmol/L Hcy-thiolactone, respectively) (Table 1) demonstrates that the effect of homocysteinylation on HDL-paraoxonase activity is related to basal HDL-PON activity.

A positive correlation was established between the percentage decrease of HDL-PON activity and the percentage increase of –SH groups ($r = 0.80$, $P < .001$, and $r = 0.76$, $P < .001$ in HDL incubated in the presence of 10 μmol/L and 1 mmol/L Hcy-thiolactone, respectively) (Table 1). These results suggest that the modification of PON activity in Hcy-HDL are likely related to the changes of the levels of −SH groups of HDL.

DISCUSSION

Recent studies 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 homocysteinylated proteins.[11,12] Homocysteinylation of plasma proteins[11,12] and lipoproteins[22,27] can be induced in vitro by incubation with Hcy-thiolactone; therefore, lipoproteins incubated with different concentrations of Hcy-thio-

Table 1. Correlation Coefficients (*r*) Between HDL-PON Activity and Percentage Increase of −SH Groups in Hcy-HDL

Correlation	Hcy-Thiolactone (10 μmol/L)	Hcy-Thiolactone (1 mmol/L)
Basal HDL-PON actvity *v* % increase −SH groups in Hcy-HDL	$r = -0.73$, $P < .001$ (n = 16)	$r = -0.70$, $P < .002$ (n = 16)
Basal HDL-PON activity *v* % decrease HDL-PON activity in Hcy-HDL	$r = -0.76$, $P < .001$ (n = 16)	$r = -0.86$, $P < .001$ (n = 16)
% decrease HDL-PON activity *v* % increase −SH groups in Hcy-HDL	$r = -0.80$, $P < .0001$ (n = 16)	$r = 0.76$, $P < .0001$ (n = 16)

lactone represent a useful experimental model to study the structural and functional alterations of homocysteinylated lipoproteins.

The significant increase in −SH groups in Hcy-HDL, observed in our experimental conditions, demonstrates that homocysteinylation occurs in vitro in HDL incubated in the presence of increasing concentrations of Hcy-thiolactone. The negative correlation established between the basal levels of HDL-PON activity versus the percentage increase of −SH groups in Hcy-HDL suggests a relationship between the susceptibility of HDL to homocysteinylation and basal HDL-PON activity. Therefore, we suggest that HDL with higher HDL-PON activity could supply better protection against protein homocysteinylation than HDL with lower HDL-PON activity. These findings are in agreement with recent studies; in fact, Jakubowski et al[19] showed that paraoxonase activity correlates with HTase activity and high activity forms of Htase afford better protection against the reaction of homocysteinylation of serum proteins. Moreover, Billecke et al[28] demonstrated that purified human PON 1 R isozyme hydrolyzes homocysteine thiolactone more efficiently than the Q isozyme.

Gender- and age-dependent modifications of paraoxonase activity and age-related changes of the susceptibility of HDL to lipid peroxidation have been previously described.[29,30] However, only male young normolipemic subjects were included in the present study; we can therefore exclude that the differences in susceptibility to homocysteinylation are age- or sex-related.

Using model peptides and proteins it was shown that the sensibility of proteins to homocysteinylation is mainly related to the levels of $-NH_2$ groups.[31]

The levels of $-NH_2$ groups were slightly higher in L-HDL-PON with respect to H-HDL-PON; therefore, the differences in susceptibility of HDL could be related also to the differences in the accessibility of $-NH_2$ groups (data not shown).

Our results show also a significant decrease of PON activity in Hcy-HDL. The negative correlation established between the basal values of HDL-PON activity and the percentage decrease of HDL-PON activity after the incubation with Hcy-thiolactone suggests that subjects with higher values of basal HDL-PON activity have a lower decrease in PON activity after homocysteinylation with respect to subjects with lower HDL-PON activity.

Some hypotheses can be advanced to explain the decreased activity of PON in Hcy-HDL. Previous studies demonstrated that PON is a lipid-dependent enzyme[32] and that HDL-PON is highly sensitive to compositional changes of lipid and/or apoprotein of HDL[33,34] and to modifications of physico-chemical properties as demonstrated by the significant decrease of HDL-PON in oxidized HDL[15,35] and/or glycated HDL.[34] We suggest that, in our experimental conditions, the decrease of PON activity in Hcy-HDL, in the absence of modification of lipid peroxidation, is likely related to compositional changes induced by homocysteinylation. This hypothesis is supported by the statistically significant correlation between the percentage de-

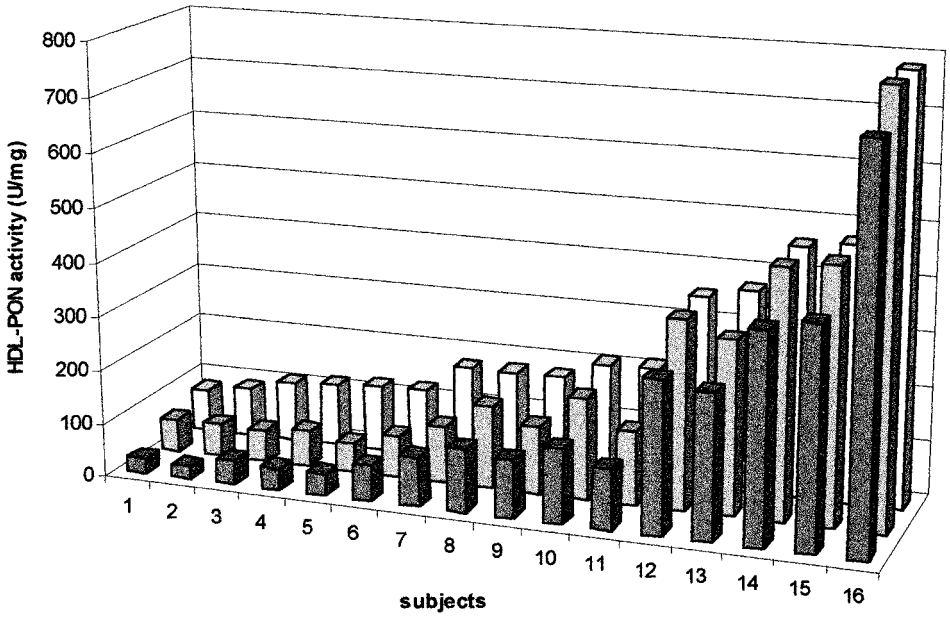

Fig 4. Levels of PON activity in basal HDL (□) and in Hcy-HDL (■, HDL incubated with 10 μmol/L Hcy-thiolactone; ■, HDL incubated with 1mmol/L Hcy-thiolactone) (n = 16).

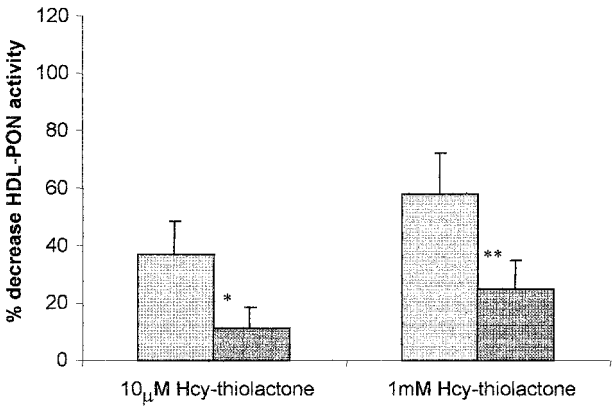

Fig 5. Percentage decrease of PON activity in HDL after incubation with 10 μmol/L and 1 mmol/L Hcy-thiolactone. Subjects with HDL-PON activity below (■) or above (■) the median value (178.5U/mg). Results are presented as percentage decrease with respect to control HDL. *$P < .001$ *v* HDL with PON activity below the median value; **$P < .001$ *v* HDL with PON activity below the median value.

crease of HDL-PON activity and the percentage increase of −SH groups in Hcy-HDL. The increase in −SH groups could reflect in structural modifications of HDL-apoproteins and/or modifications of the interactions PON-phospholipids and/or PON-apoprotein interaction at the lipoprotein surface of Hcy-HDL.

The metabolic relationship between Hcy and Hcy-thiolactone and the molecular mechanism by which these molecules are involved in the development of human diseases deserve of further studies. Increased levels of Hcy were correlated with premature atherosclerosis and thrombosis[2,5] and this was suggested to reflect the homocysteinylation of aminogroups of LDL.[22] An initial finding of high levels of Hcy-thiolactone in human serum[36] contrasts with later reports noting its apparent absence in human serum due to the presence of a thiolactonase activity.[13] However, the effect of Hcy-thiolactone was observed also at low concentrations (10 μmol/L)[7] and it was suggested that even modestly low levels of Hcy-thiolactone might occur in homocysteinuria.

Previous studies by us and other studies[15,34] demonstrated that susceptibility of HDL to peroxidation is inversely correlated with HDL-PON activity, suggesting that subjects with low activity are more exposed to oxidative stress than subjects with higher activity. The present study demonstrates that the basal PON activity modulates also the susceptibility of HDL to homocysteinylation and the effect of homocysteinylation on HDL-PON activity. The enzyme PON plays an important role against the development of atherosclerosis due to its ability of protect LDL[14] and HDL[15] from lipid peroxidation; moreover, by detoxifying Hcy-thiolactone, paraoxonase/Htase would protect proteins against homocysteinylation, another potential contributing factor to atherosclerosis.

As far as concerns the pathophysiological relevance of our results, a decrease in PON activity was demonstrated in several human disease as diabetes,[37] Alzheimers, and vascular dementia.[38] Moreover, dietary-induced changes have been recently described.[39]

In conclusion, genetic and dietary factors modulate PON activity; we suggest that subjects with low HDL-paraoxonase activity could be more exposed to disease involving oxidative damage and to the toxic effect of Hcy-thiolactone. Further studies are necessary to elucidate whether homocysteinylation is an atherogenic modification and whether other key functions of HDL such as reverse cholesterol transport and the ability to protect LDL against oxidative damage are modified after homocysteinylation.

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