

The Antiatherogenic Function of HDL Is Impaired in Hyperhomocysteinemic Subjects^{1,2}

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Abstract

High plasma homocysteine concentrations have been associated with increased risk of cardiovascular disease, whereas plasma HDL concentration is inversely correlated to such disorders. We hypothesized that hyperhomocysteinemic subjects may have dysfunctional HDL. We therefore investigated the ability of serum from hyperhomocysteinemic male and female subjects ($n = 10$) and control subjects ($n = 10$) to induce cholesterol efflux and to inhibit release of inflammatory mediators from human umbilical vein endothelial cell. We found that serum from hyperhomocysteinemic subjects had impaired ability to induce cholesterol efflux from lipid-loaded macrophages compared with healthy controls. HDL from those with markedly raised homocysteine concentrations had a reduced antiinflammatory effect in tumor necrosis factor- α -activated endothelial cells with an attenuated suppressive effect on interleukin-6 growth-related oncogene- α release. Also, the activity of paraoxonase in serum, a multifunctional enzyme with antioxidative effects in relation to the function of HDL, was significantly reduced in hyperhomocysteinemic subjects, in particular those with markedly raised homocysteine concentration. Our findings suggest that hyperhomocysteinemic individuals have dysfunctional HDL particles with attenuated antiatherogenic activity and may represent a novel explanation for the increased risk of cardiovascular events in these individuals. *J. Nutr.* 138: 2070–2075, 2008.

Introduction

High plasma homocysteine concentrations have been associated with increased risk of cardiovascular diseases (CVD)⁷ (1–3). However, several large randomized clinically controlled trials have now shown that lowering homocysteine with vitamin B therapy does not reduce the risk of CVD or mortality among subjects with known CVD (4–6). Impaired endothelial function, increased oxidative stress, alterations of lipid metabolism, and induction of thrombosis have been suggested to be pathogenic links between hyperhomocysteinemia and CVD. However, the mechanism by which homocysteine could promote atherogenesis and thromboembolic disorders is far

from clear and the role of homocysteine in human CVD is still debated.

Epidemiological studies have shown that plasma HDL concentration is inversely correlated to increased risk of CVD (7–9). The mechanistic relationship between low plasma HDL and atherosclerosis has not been fully elucidated but involves the ability of HDL to promote reverse cholesterol transport from peripheral tissue to the liver as well as its antiinflammatory properties. Because low plasma HDL concentration sometimes is associated with increased risk of CVD (7–9), whereas other conditions with low plasma HDL concentration are associated with improved prognosis [e.g. being a carrier of the apolipoprotein (apo)A-1 Milano] (10), it seems that it is not only the concentration per se but also the function of the HDL particles that is important for its antiatherogenic effects. In fact, it has been suggested that it might be more important to improve HDL metabolism, composition, and function than to merely increase the HDL concentrations.

HDL particles are susceptible to structural modifications mediated by various mechanisms, including oxidation, glycation, or enzymatic degradation, affecting their functional properties (11). Moreover, recent *in vitro* studies have shown that homocysteinylation of HDL may reduce the activity of the enzyme paraoxonase (PON), which is associated with human HDL, thus rendering the HDL particle more susceptible to oxidative damage (12). It has recently also been shown that

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⁷ Abbreviations used: apo, apolipoprotein; CVD, cardiovascular disease; d, density; FCS, fetal calf serum; GRO, growth-related oncogene; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, monocyte chemoattractant protein; PON, paraoxonase; THP-1, Tamm-Horsfall Protein 1; TNF α , tumor necrosis factor- α .

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HDL is modulated by systemic inflammation (13) and, interestingly, during various inflammatory conditions, HDL itself may become inflammatory rather than antiinflammatory.

Based on previous reports on an inflammatory phenotype in hyperhomocysteinemic subjects (14–16), we hypothesized that these subjects may have a dysfunctional HDL, potentially affecting the HDL-mediated reverse cholesterol transport and the HDL-mediated antiinflammatory function. We therefore investigated: 1) the ability of serum from hyperhomocysteinemic individuals to promote cholesterol efflux from lipid-loaded macrophages; 2) the ability of HDL isolated from hyperhomocysteinemic individuals to inhibit cytokine-induced expression of inflammatory markers in endothelial cells; and 3) the activity of PON, a multifunctional enzyme with antioxidative effects in serum in subjects with hyperhomocysteinemia and in healthy controls.

Methods

Subjects. Ten subjects with fasting plasma total homocysteine concentration ≥ 15 $\mu\text{mol/L}$ were recruited at the Lipid Clinic and the Institute for Clinical Biochemistry, Rikshospitalet University Hospital. Five of the hyperhomocysteinemic subjects were currently on statin treatment (Table 1). Control subjects were 10 age- and sex-matched healthy control volunteers with no history of hypertension, diabetes, CVD, or other acute or chronic illness. The study protocol was approved by the Regional Committee of Medical Ethics and the investigation conforms with the principles outlined in the Declaration of Helsinki. Informed consent was obtained from all subjects. Plasma and serum samples were collected after an overnight fast and stored at -80°C until analysis as previously described (17).

Isolation of lipoproteins (LDL and HDL) and oxidation of LDL. LDL [density (d) 1.019–1.063 kg/L], HDL₂ (d 1.063–1.125 kg/L), and HDL₃ (d 1.125–1.210 kg/L) from 1 healthy control subject, and HDL (d 1.063–1.1210 kg/L) from 5 hyperhomocysteinemic subjects (1 female and 4 males, 51 [29–69] y, homocysteine concentration 28 [23–42] $\mu\text{mol/L}$), and 3 healthy subjects (1 female and 2 males, 38 [38–39] y, homocysteine concentration 10 [5–10] $\mu\text{mol/L}$), with comparable levels of total cholesterol, LDL, HDL, apoA-1, and apoB were isolated by sequential ultracentrifugation at 8°C in a TI 80 rotor (Beckman Optima LE-80K ultracentrifuge) as described before (17). LDL was oxidized in the presence of Cu^{2+} (10 $\mu\text{mol/L}$) for 24 h as previously described (17). HDL were homocysteinylated according to Ferretti et al. (12). Briefly, HDL

(100 μg), resuspended in PBS (pH 8.2), were incubated at 25°C in the presence or absence of homocysteine-thiolactone (100 $\mu\text{mol/L}$ or 1 mmol/L; Sigma) for 1 h. After incubation the solution was passed through a Sephadex G-25 column, equilibrated with PBS to separate the unbound homocysteine-thiolactone from HDL.

Cell cultures. Primary human umbilical vein endothelial cells (HUVEC) were isolated and cultured in endothelial cell growth medium as previously described (17). After various time points, cell-free supernatants or cell lysates (cell pellets were treated in 0.1% Triton X-100 lysis buffer and supernatant was collected after centrifugation) were stored at -80°C until further analyses. Human monocytic leukemia cells [Tamm-Horsfall Protein 1 (THP-1) cells, art. no TIB-202] were purchased from the American Type Culture Collection (Rockville) and seeded into 24-well plates (750,000 cells per well) cultured in RPMI 1640 medium containing glutamine (4 mmol/L), penicillin (50 kU/L), streptomycin (20 mg/L), and fetal calf serum (FCS; 10% final concentration; Gibco). Cells were differentiated into macrophages by incubation for 72 h with phorbol 12-myristate 13-acetate (100 nmol/L; Sigma Chemical). Cell viability of HUVEC and THP-1 macrophages was assessed using phase contrast microscopy to evaluate the morphology before and after the various experiments.

Cholesterol efflux. THP-1 macrophages were lipid loaded by incubation with oxidized LDL (20 mg/L) in growth medium (RPMI 1640 medium with FCS) and 0.5 mCi/L (18.5 MBq/L) [^3H]-cholesterol (American Radiolabel Chemicals) dissolved in ethanol was added. After 24 h, radiolabeled media were removed and the foam cells were washed twice with 0.2% bovine serum albumin (wt:v) in RPMI. Then serum from hyperhomocysteinemic individuals or healthy controls (final concentration 10%), HDL₂, or HDL₃ (final concentrations 50 mg/L; homocysteinylated and nonhomocysteinylated) were added in RPMI 1640 medium (without FCS) and incubated for 3 h. Thereafter, the cell medium was collected and the cells were harvested in 0.2 mol/L NaOH. The radioactivity was measured by liquid scintillation counting using TRI-CARB 2300 TR Scintillation Counter (Packard). Data are presented as fractional (%) cholesterol efflux calculated as $[\text{Bq}(\text{media})]/(\text{Bq}(\text{media} + \text{cell-associated})) \times 100$.

PON enzymatic activity. We measured PON activity according to the manufacturer's instructions using EnzChek Paraoxonase Assay kit (Invitrogen), which is a highly sensitive, homogenous fluorometric assay for the organophosphatase activity of PON.

Enzyme immunoassay. Concentrations of monocyte chemoattractant protein (MCP)-1, growth-related oncogene (GRO)- α , interleukin (IL)-6, and intercellular adhesion molecule (ICAM-1) were measured in cell-free

TABLE 1 Characteristics of participants^{1,2}

	Control subjects	Hyperhomocysteinemic subjects	Extremely hyperhomocysteinemic subjects	Moderately hyperhomocysteinemic subjects
<i>n</i>	10	10	5	5
Age, <i>y</i>	43 (35–69)	49 (25–65)	48 (25–62)	50 (35–65)
Male, <i>n</i>	6	6	3	3
Statin treatment, <i>n</i>	0	5	3	2
Total cholesterol, <i>mmol/L</i>	5.4 (3.6–5.7)	4.5 (3.5–7.8)	4.5 (3.5–7.8)	4.4 (3.9–6.9)
LDL cholesterol, <i>mmol/L</i>	3.6 (2.6–4.6)	2.5 (1.7–5.5)	2.2 (1.7–5.5)	2.6 (2.1–4.6)
HDL cholesterol, <i>mmol/L</i>	1.5 (0.8–2.8)	1.3 (0.7–1.7)	1.2 (0.7–1.7)	1.5 (1.0–1.7)
ApoB, <i>mmol/L</i>	0.9 (0.7–1.2)	0.9 (0.6–1.2)	0.9 (0.6–0.9)	0.9 (0.6–1.2)
ApoA-1, <i>mmol/L</i>	1.4 (1.1–2.2)	1.5 (1.1–1.8)	1.2 (1.1–1.6)	1.5 (1.2–1.8)
Triglycerides, <i>mmol/L</i>	0.9 (0.4–1.7)	1.5 (0.4–6.0)**	1.4 (0.7–6.0)†	1.6 (0.4–2.7)
Homocysteine, $\mu\text{mol/L}$	10 (9–13)	44 (19–94)*	69 (50–94)**	22 (19–37)**
Folate, <i>nmol/L</i>	14.3 (7.9–19.9)	7.8 (3.6–14.5)**	6.6 (3.6–10.3)**	9.1 (3.6–14.5)

¹ Data are medians (min-max). Asterisks indicate different from control: * $P < 0.001$, ** $P < 0.05$, † $P = 0.053$.

² Folate was measured in serum and the other laboratory markers were measured in plasma.

supernatants (MCP-1, GRO α , IL-6) or cell lysate (ICAM-1) of HUVEC by enzyme immunoassay obtained from R&D Systems and related to the protein concentration as assessed by bicinchoninic acid protein assay (Pierce) using bovine serum albumin as standard.

Miscellaneous. C-reactive protein was measured in serum using a high-sensitivity, particle-enhanced immunoturbidimetric assay on a Modular platform (Roche Diagnostics). Concentrations of homocysteine, folate, vitamin B-12, total cholesterol, LDL cholesterol, HDL cholesterol, apoA-1, and apoB were measured in plasma or serum using routine laboratory methods (18).

Statistical analysis. For comparisons of 2 groups of individuals, the Mann-Whitney U test was used. When more than 2 groups were compared, we used the Kruskal-Wallis test. If a significant difference was found, the Mann-Whitney U test was used to determine the differences between each pair of groups. We calculated Spearman rank order correlation coefficients with the entire study population included in each analysis to evaluate relationships between different variables. Differences were considered significant at $P < 0.05$ for all analyses. SPSS 11.0 for Windows was used for statistical analysis. Data are given as median (minimum-maximum) unless otherwise stated.

Results

Baseline characteristics. Circulating homocysteine, folate, and triglyceride concentrations were higher in hyperhomocysteinemic patients compared with controls (Table 1). A similar pattern was found when those that were taking statin therapy were excluded from the calculations (data not shown).

Cholesterol efflux. To test if HDL particles from hyperhomocysteinemic individuals, operating in an inflammatory microenvironment, had altered function, we examined the ability of serum from the hyperhomocysteinemic subjects to induce cholesterol efflux from cultured lipid-loaded macrophages. Serum from hyperhomocysteinemic subjects had impaired ability to induce cholesterol efflux from lipid-loaded macrophages compared with healthy controls ($P < 0.05$) (Fig. 1A). We then subdivided the hyperhomocysteinemic subjects into 2 groups, depending on the plasma homocysteine concentrations (above or below plasma median homocysteine concentration of 44 $\mu\text{mol/L}$), resulting in 2 subgroups of individuals: group 1, extreme hyperhomocysteinemic subjects with markedly elevated plasma homocysteine concentrations (69 [50–94] $\mu\text{mol/L}$; $n = 5$); and group 2, hyperhomocysteinemic subjects with moderately elevated homocysteine concentrations (22 [19–37] $\mu\text{mol/L}$; $n = 5$), in addition to the control subjects (10 [9–13] $\mu\text{mol/L}$; $n = 10$) (Table 1). Although plasma apoA-1 and HDL concentrations did not differ among these 3 groups of individuals (Table 1), serum from both groups of hyperhomocysteinemic subjects had impaired ability to induce cholesterol efflux from lipid-loaded macrophages compared with healthy controls ($P \leq 0.05$) (Fig. 1B). Notably, the ability of serum to induce cholesterol efflux

was inversely correlated with the concentrations of homocysteine ($r = -0.61$; $P = 0.008$) and triglycerides ($r = -0.48$; $P = 0.039$).

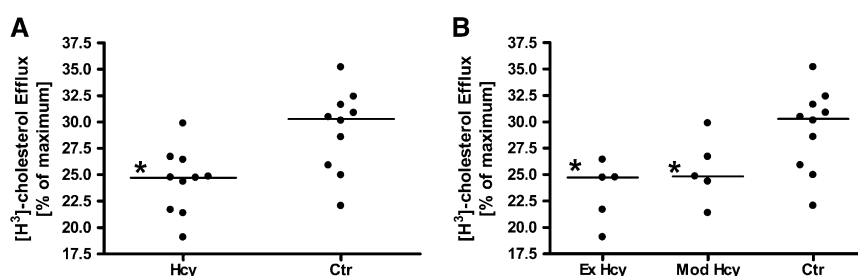
To investigate whether homocysteinylation of HDL₂ and HDL₃ in vitro could be important for the ability of HDL to induce cholesterol efflux, we subsequently homocysteinylated HDL₂, HDL₃, and serum from healthy subjects in vitro and examined if these modulations influenced the ability of HDL or serum to induce cholesterol efflux. However, we found no significant effect on homocysteinylation of HDL₂, HDL₃, or serum from healthy controls on the ability to promote cholesterol efflux (data not shown), suggesting that this in vitro procedure could not mimic the situation in HDL particles from hyperhomocysteinemic individuals being persistently exposed to high concentrations of homocysteine in an inflammatory microenvironment.

Antiinflammatory effects of HDL. To study if HDL in hyperhomocysteinemic subjects also had an impaired antiinflammatory potential, we first isolated HDL from 5 additional hyperhomocysteinemic subjects. When examining the ability of HDL from these individuals to inhibit the tumor necrosis factor- α (TNF α)-induced release of GRO α , IL-6, MCP-1, and the expression of ICAM-1 in HUVEC, mimicking the ability of HDL to modulate vascular inflammation, several findings were revealed. First, whereas HDL from healthy controls impaired the release of GRO α from TNF α -activated HUVEC ($P \leq 0.05$) (Fig. 2A), this effect on GRO α was attenuated in those with hyperhomocysteinemia. Second, the inhibitory effect of HDL isolated from the hyperhomocysteinemic individuals on the release of IL-6 was less than that of HDL isolated from healthy controls ($P < 0.05$) (Fig. 2B).

PON activity. PON, an enzyme with antioxidative properties, has previously been shown to be involved in antiatherogenic effects of HDL, influencing both its effect on cholesterol efflux and inflammation (19). Subjects with hyperhomocysteinemia had markedly lower PON activity compared with healthy controls ($P < 0.05$; Fig. 3A). When we subdivided the hyperhomocysteinemic subjects as described above, those with extreme hyperhomocysteinemia had markedly lower PON activity compared with both healthy controls and those with moderately increased homocysteine concentrations ($P < 0.05$) (Fig. 3B). Moreover, PON activity was negatively correlated to plasma homocysteine concentrations ($r = -0.620$; $P = 0.006$) and plasma triglycerides ($r = -0.481$; $P = 0.043$) and positively correlated to the ability of serum to promote cholesterol efflux ($r = 0.437$; $P = 0.061$) and to serum folate concentrations ($r = 0.655$; $P = 0.004$).

Effects of statins and triglycerides. The use of statins and high concentrations of triglycerides in subjects with hyperhomocysteinemia could represent confounders when interpreting our data. However, hyperhomocysteinemic statin users and hyperhomocysteinemic nonstatin users did not differ in any of the

FIGURE 1 Cholesterol efflux from lipid-loaded THP-1 macrophages incubated with serum from hyperhomocysteinemic participants (Hcy; $n = 10$) and healthy controls (Ctr; $n = 10$) (A) and from extremely hyperhomocysteinemic participants (Ex Hcy, $n = 5$), moderately hyperhomocysteinemic participants (Mod Hcy, $n = 5$) and healthy controls (Ctr, $n = 10$) (B). Points are individual values and horizontal lines indicate medians. *Different from Ctr, $P \leq 0.05$.



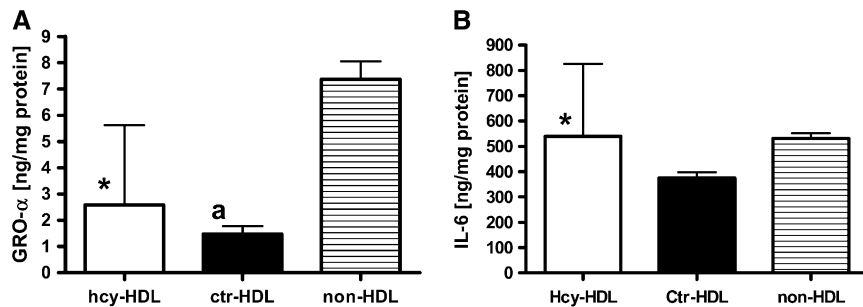


FIGURE 2 Antiinflammatory effects of HDL in endothelial cells isolated from hyperhomocysteinemic subjects (Hcy, $n = 5$) and healthy controls (Ctr, $n = 3$), on the release of GRO α (A) and in IL-6 (B) from TNF α (100 μ g/L) stimulated HUVEC after culturing for 20 h. Values are median (range). *Different from Ctr-HDL, $P \leq 0.05$, ^adifferent from non-HDL, $P \leq 0.05$.

variables measured (data not shown). Nevertheless, relatively few patients were studied and because statin treatment has been shown to increase PON activity (20–22), we cannot exclude that we have underestimated the difference in PON activity between hyperhomocysteinemic individuals and healthy controls. Increased concentrations of triglycerides have been shown to reduce cholesterol efflux and in the present study, the impaired ability of serum to induce cholesterol efflux was inversely correlated with triglyceride concentrations (see above; $r = -0.48$; $P = 0.039$). However, for PON activity, those with extreme hyperhomocysteinemia had lower activity ($P \leq 0.05$) compared with the subjects with moderate hyperhomocysteinemia and, importantly, those with extreme and moderate hyperhomocysteinemia differed only in homocysteine concentration, not in any of the lipids measured (Table 1). Whereas these data suggest that our findings in the hyperhomocysteinemic group do not merely reflect that these patients also have raised triglyceride concentrations, triglycerides may be a confounder in the interpretation of our data.

Discussion

HDL have been shown to protect against atherosclerosis (7–9). The precise mechanisms remain unclear, but the ability of HDL to promote efflux of cholesterol from the cells in the arterial wall, as well as its antiinflammatory properties, is considered to be of major importance (23). Evidence indicates that hyperhomocysteinemia, which occurs in ~5–7% of the general population, is a risk factor for CVD, but how homocysteine mediates its potential proatherogenic effects is still debated. Whereas epidemiological data indicate that elevation of plasma homocysteine is not associated with a significant change in plasma total cholesterol, some studies have reported a negative correlation with HDL concentrations (23,24). Moreover, Mikael et al. (23) recently suggested that the decrease in plasma HDL in humans and animals with hyperhomocysteinemia is caused in part by decreased hepatic expression of apoA-1. In the present study, we extend these findings by showing that individuals with hyperhomocysteinemia have altered function of HDL particles as

shown by their impaired ability to promote cholesterol efflux from lipid-loaded macrophages as well as by their impaired antiinflammatory capacity in endothelial cells. In fact, HDL from individuals with markedly increased homocysteine concentrations had attenuated suppressing effects on the TNF α -induced release of IL-6 in HUVEC. It is not inconceivable that this altered function of HDL particles in relation to hyperhomocysteinemia could contribute to the increased risk of cardiovascular risk in these individuals.

The antiatherosclerotic function of HDL is at least partly ascribed to its role in reverse cholesterol transport and requires the integrity of HDL structure. In this study, we show an impaired ability of serum from individuals with markedly raised homocysteine concentrations, and with HDL and ApoA-1 concentrations within normal limit, to promote cholesterol efflux from lipid-loaded macrophages. Although other factors could contribute to the impaired serum-induced cholesterol efflux (e.g. altered function of ATP-binding cassette transporter A1), our findings suggest impaired HDL function in hyperhomocysteinemic individuals. Notably, homocysteinylated HDL₂ and HDL₃ in vitro did not influence their ability to promote cholesterol efflux, suggesting that short-time exposure to homocysteine cannot mimic the in vivo situation in serum from individuals with hyperhomocysteinemia. However, although our findings suggest that hyperhomocysteinemic subjects have impaired HDL-mediated reverse cholesterol efflux in macrophages, future studies should also investigate other mechanisms that could influence cholesterol efflux in these individuals (e.g. liver clearance, bile excretion, and intestinal reabsorption).

HDL particles possess antiinflammatory properties at least partly related to their antioxidative activities. In this study, we confirm such antiinflammatory activities of HDL from healthy individuals in TNF α -activated endothelial cells. However, these antiinflammatory effects of HDL were markedly attenuated in individuals with raised homocysteine concentrations. There are several reports on alterations in HDL composition during inflammation leading to attenuated antiinflammatory activities or even inflammatory properties of HDL (11,13,25), and in the present study, we report similar alteration of HDL function in individuals

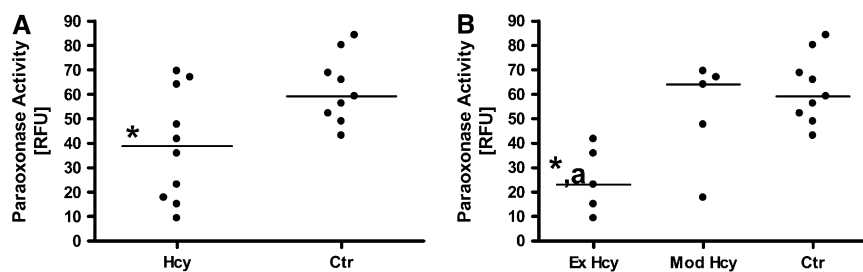


FIGURE 3 Activity of PON in serum from hyperhomocysteinemic subjects (Hcy; $n = 10$) and healthy controls (Ctr; $n = 10$) (A) from extremely hyperhomocysteinemic subjects (Ex Hcy, $n = 5$), moderately hyperhomocysteinemic subjects (Mod Hcy, $n = 5$), and healthy controls (Ctr, $n = 9$) (B). Points are individual values and horizontal lines indicate medians. *Different from control, $P < 0.05$, ^adifferent from Mod Hcy, $P \leq 0.05$.

with hyperhomocysteinemia. Interestingly, the attenuated anti-inflammatory effects of HDL in individuals with hyperhomocysteinemia seem not to include all responses. Thus, whereas HDL particles from these individuals suppressed ICAM-1 concentrations in TNF α -activated HUVEC, they had an attenuated response on the release of IL-6 and GRO α in these cells, underscoring the complexity of these HDL-mediated effects. Nevertheless, whereas an attenuated antiinflammatory HDL response could be part of an evolutionary conserved mechanism of nonspecific innate immunity aimed to protect against infections, inflammatory effects of HDL would clearly be harmful in relation to vascular inflammation during atherogenesis, possibly representing a causal link between raised homocysteine concentrations and atherosclerotic disorders.

Formation of inflammatory HDL has been suggested to correlate with decreases in the activities of various HDL-associated enzymes (25), such as PON, an multifunctional enzyme with antioxidant capacity (26), and the ability to detoxify the homocysteine metabolite homocysteine thiolactone (27). Indeed, we found that the hyperhomocysteinemic subjects, and particularly those with extreme hyperhomocysteinemia, were characterized by significantly less PON activity than healthy controls. Moreover, the PON activity was negatively correlated to plasma homocysteine concentrations and to the ability of serum to promote cholesterol efflux, further suggesting a link between PON activity and impaired HDL function during hyperhomocysteinemia. Thus, although we have no mechanistic data, it is not inconceivable that hyperhomocysteinemia itself could attenuate PON activity. Homocysteine has previously been shown to be an independent predictor of PON activity (28), further supporting such a notion. However, the relationship could also be more indirect. We have previously shown that hyperhomocysteinemic subjects are characterized by enhanced inflammatory responses (14–16). Based on the ability of inflammation to downregulate PON expression, it is possible that the attenuated PON activity in hyperhomocysteinemic subjects could at least partly reflect their inflammatory phenotype.

The present study has certain limitations, such as the low number of individuals and the possibility that statin use and triglycerides may represent confounders when interpreting our data. Thus, our data will have to be confirmed in a larger study population before drawing any firm conclusion. Nevertheless, although the data should be interpreted with caution, our findings suggest dysfunctional HDL particles in individuals with hyperhomocysteinemia, affecting the ability of HDL to promote cellular cholesterol efflux as well as its antiinflammatory properties. These changes in HDL function during hyperhomocysteinemia may attenuate the antiatherogenic function of these particles, possibly representing a novel explanation for the increased risk of cardiovascular events in these individuals. However, some of our findings (e.g. decreased PON activity) were particularly pronounced in those with markedly raised homocysteine concentrations (>50 μ mol/L), suggesting that some of the detrimental effects of homocysteine may be present only when the subjects are truly hyperhomocysteinemic. This may be relevant in some studies that have failed to show detrimental effects of homocysteine in populations with normal or only moderately raised homocysteine concentrations.

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