Hyperhomocysteinemic Subjects Have Enhanced Expression of Lectin-Like Oxidized LDL Receptor-1 in Mononuclear Cells

Kirsten B. Holven,*†** Hanne Scholz,** Bente Halvorsen,** Pål Aukrust,**‡ Leiv Ose* and Marit S. Nenseter†*‡

*The Lipid Clinic, †MSD Cardiovascular Research Center, ‡Research Institute for Internal Medicine and §Section of Clinical Immunology and Infection Diseases, Medical Department, Rikshospitalet University Hospital, Oslo, Norway

ABSTRACT An elevated plasma concentration of homocysteine is an independent risk factor for cardiovascular disease. However, the mechanisms are still unclear. Lectin-like oxidized LDL receptor-1 (LOX-1) has ligand specificity for oxidized LDL (oxLDL). We hypothesized that homocysteine’s atherogenic effects may involve LOX-1-mediated mechanisms. We examined the effect of folic acid supplementation for 6 wk and 12 mo (5 mg/d for 1 wk, 1 mg/d for 37 wk and 0.4 mg/d for the remaining 14 wk) on LOX-1 mRNA levels and on oxLDL-induced release of tumor necrosis factor α from peripheral blood mononuclear cells in hyperhomocysteinemic individuals. Compared with healthy controls, hyperhomocysteinemic subjects had elevated mRNA levels of LOX-1 in mononuclear cells (P < 0.001), and their mononuclear cells released more tumor necrosis factor α (TNFα) upon oxLDL stimulation (P = 0.01). This oxLDL-stimulated release of TNFα correlated with LOX-1 expression (r = 0.57, P = 0.026). Folic acid treatment led to a normalization of homocysteine levels accompanied by a reduction in LOX-1 gene expression (P < 0.02) and in oxLDL-stimulated release of TNFα (P < 0.05). These novel findings suggest both that homocysteine exerts its atherogenic effect in part by elevating levels of LOX-1, thereby enhancing oxLDL-induced inflammatory responses, and most important, that folic acid supplementation may down-regulate these responses. J. Nutr. 133: 3588–3591, 2003.

KEY WORDS: • homocysteine • folic acid • chemokines • PBMC • inflammation

Multiple epidemiological studies have indicated that elevated plasma levels of homocysteine portend increased risk of cardiovascular disease (1–2). Hyperhomocysteinemia may be caused by defects in enzymes involved in homocysteine metabolism or by low intake of fruits and vegetables rich in B-vitamins. The precise mechanism has yet to be fully defined (3–4).

There is emerging evidence that oxidized LDL (oxLDL)3 play an important role in the pathogenesis of atherosclerosis (5). Thus, oxLDL may cause lipid accumulation and foam cell formation, and elicit inflammatory changes and apoptosis (6). Different molecules are involved in the uptake of oxLDL into cells, such as scavenger receptor A, CD36 and scavenger receptor B1; recently, lectin-like oxidized LDL receptor-1 (LOX-1) has attracted particular interest (7–10). Although originally identified as a receptor predominantly expressed in endothelial cells, LOX-1 is also expressed in other cells such as macrophages, dendritic cells, cardiomyocytes and vascular smooth muscle cells (11). Moreover, LOX-1 is highly expressed in atherosclerotic lesions of human carotid arteries, suggesting that LOX-1 may be implicated in initiation and progression of atherosclerosis, possibly also involving inflammatory mechanisms (12).

Gene expression of LOX-1 is induced by oxidative stress, inflammation and mechanical stimuli. In the present study we hypothesize that homocysteine may exert its atherogenic effect in part through LOX-1-mediated mechanisms. We therefore examined the effect of folic acid supplementation on LOX-1 expression in peripheral blood mononuclear cells (PBMC) from patients with hyperhomocysteinemia and whether any modulation of LOX-1 expression was associated with any changes in the oxLDL-stimulated release of inflammatory cytokines.

SUBJECTS AND METHODS

Subjects. Adult subjects < 75 y of age (n = 9; 3 female; median age 47 y) with hyperhomocysteinemia (fasting plasma homocysteine concentration > 15 μmol/L), were recruited at the Lipid Clinic, Rikshospitalet University Hospital, Oslo, Norway. The study protocol was approved by the Regional Committee of Medical Ethics and by the Norwegian Medicine Control Authorities. Informed consent was obtained from all patients. Eight of the patients had hypercholesterolemia, four were on statin treatment, and seven were current smokers. The subjects had no history of hypertension, diabetes or coronary heart disease. For comparison, blood samples were also collected from sex- and age-matched nonsmoking healthy controls (n = 9; 4 female; median age 47 y). Control subjects (n = 8) in the oxLDL experiment (see below) were from our pool of healthy blood donors (30% female; mean age = 40 y).

Folic acid treatment. The study design has been reported previously (13). Briefly, subjects with hyperhomocysteinemia received folic acid on the following schedule: 5 mg/d for the first week to ensure rapid homocysteine-lowering effect, 1 mg/d for the next 37 wk and 0.4 mg/d as a maintenance dose for the last 14 wk. All subjects were previously instructed by a nutritionist to follow the National...
Cholesterol Education Program Step I Diet, and all remained on this diet throughout the study. All subjects were observed for at least 6 wk, and seven of the subjects completed the study (12 mo). At baseline, and after 6 wk and 12 mo of treatment, venous blood samples were collected after an overnight fast. To minimize interassay variation, all samples from each patient were analyzed in the same run.

**Human LOX-1 mRNA expression.** Total RNA was isolated from PBMC pellets as described previously (14). To detect gene expression of human LOX-1, 100 ng total RNA from each sample was reverse-transcribed by TaqMan Reverse Transcription reagent kit (Applied Biosystems, Foster City, CA). For quantitative real-time RT-PCR amplification of LOX-1, we used a fluorogenic probe: 5'-CAGCCAGAAATCTGAAATCCTCAAGAAACACTGAAGA-3', and primer set sequence: 5'-CTGGAGGGACGATCTGACG-3' and 5'-CGGACAGGGACTGAACAT-3'. Similarly, the same samples were PCR amplified for endogenous β-actin (Predeveloped TaqMan Assay Reagents; Applied Biosystems), and LOX-1 was normalized against β-actin expression. The PCR reaction was performed in a 96-well microtiter plate on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems). Quantification was performed using the standard curve method.

**Isolation and oxidation of LDL.** The LDL were isolated and oxidized as described previously (15). The oxLDL used before and after folic acid treatment contained 266 and 349 nmol lipid peroxide per mg LDL protein, respectively, and the relative electrophoretic mobility of these LDL samples was 3.5 and 4.0, respectively. The oxLDL were stored under N2 and used within 2 wk.

**Release of tumor necrosis factor α (TNFα) from PBMC induced by oxLDL.** The PBMC were incubated in flat-bottomed 96-well trays as described previously (15). Cell-free supernatants were harvested after 24 h, divided into aliquots and stored at −80°C until TNFα analysis.

**Miscellaneous.** The TNFα was measured using a commercially available enzyme immunoassay kit (Pelikine-compact; CLB, Amsterdam, the Netherlands). Plasma homocysteine concentration was determined by HPLC (13). Serum concentrations of folate and vitamin B-12 were measured using the fluorometric assay autodelfia folate and autoDELFIA B-12, respectively (PerkinElmer, Wallac Oy, Turku, Finland). Total cholesterol, LDL and HDL cholesterol and triglycerides were measured using enzymatic colorimetric tests (Hitachi 917; Roche Diagnostics, Indianapolis, IN), creatinine was measured using kinetic colorimetric tests (Hitachi 917; Roche Diagnostics), and total and differential leukocyte counts were determined using Cell-DYN 4000 (Abbott Laboratories, Abbott Park, IL).

**Statistical analysis.** Data are given as medians and ranges. Baseline values in patients and control subjects and in statin users versus nonusers were compared by the Mann-Whitney U test. Friedmann and Wilcoxon signed ranks tests were used to examine differences within individuals over time and between stimulated versus unstimulated cells. P-values were Bonferroni-corrected. Spearman’s rank correlation coefficients were calculated to evaluate relationships between different variables. The level of statistical significance was set at P < 0.05.

**RESULTS**

**Baseline characteristics.** Hyperhomocysteinemic subjects were characterized by higher plasma concentrations of homocysteine [26 (20–52) vs. 10 (6–12) μmol/L, P < 0.001], total and LDL cholesterol [6.9 (4.0–8.7) vs. 5.1 (3.6–6.1) mmol/L and 5.2 (2.5–6.7) vs. 3.4 (2.0–4.3) mmol/L, respectively, P < 0.05], and lower folate [6.0 (3.9–8.2) vs. 12.2 (10.1–22.7) μmol/L, P < 0.001] and vitamin B-12 [210 (105–285) vs. 290 (215–355) pmol/L, P < 0.01] than healthy controls. Concentrations of HDL cholesterol, triglycerides and creatinine did not differ between groups (data not shown).

**Levels of LOX-1 mRNA in PBMC.** Patients with hyperhomocysteinemia had markedly elevated levels of LOX-1 mRNA compared to healthy control subjects (Fig. 1). Moreover, LOX-1 mRNA levels correlated positively with homocysteine concentration (r = 0.65, P = 0.004), and negatively with concentrations of folate (r = −0.62, P = 0.006) and vitamin B-12 (r = −0.72, P = 0.001).

In addition to hyperhomocysteinemia, eight of the nine patients had hypercholesterolemia. However, the LOX-1 mRNA levels [40 (2–811), arbitrary units; n = 9] did not differ from those in patients with hypercholesterolemia alone [109 (31–981) arbitrary units; n = 5; P = 0.55]. Furthermore, there were marked differences in LDL cholesterol levels within the patient population, reflecting the use of statin by four of the nine hyperhomocysteinemic patients. However, LOX-1 expression did not differ between statin users and nonusers [36.5 (12–364) vs. 384 (2–811), arbitrary units; P = 0.46].

**Induction of TNFα in PBMC by oxLDL.** The oxLDL-activated PBMC released more TNFα than unstimulated cells (Fig. 2), and this oxLDL-stimulated release correlated with the LOX-1 gene expression in these cells (r = 0.57, P = 0.026). In contrast, PBMC from healthy controls released very low levels of TNFα, not only in unstimulated cells, but also after oxLDL-stimulation. In fact, unstimulated and stimulated release of TNFα in the controls was below the detection limit of EIA in 6 of 8 subjects.

**Effect of folic acid treatment on LOX-1 mRNA levels and release of TNFα.** Folic acid treatment reduced plasma homocysteine levels [26 (20–52) vs. 10 (8–13) and 12 (7–26) μmol/L, P < 0.04 after 6 wk and 12 mo, respectively], and increased plasma folate levels [6.0 (3.9–8.2) vs. 37.9 (16.6–113) and 30.9 (10.0–50) μmol/L, P < 0.04, after 6 wk and 12 mo, respectively]. Folic acid treatment did not affect plasma total, LDL and HDL cholesterol, vitamin B-12, creatinine and leukocyte count (data not shown).

The decrease in homocysteine levels was accompanied by a marked decrease in LOX-1 mRNA expression, reaching levels not different from those in healthy controls (Fig. 1). Moreover, folic acid treatment significantly reduced the oxLDL-stimulated release of TNFα in these cells after 6 wk of therapy (Fig. 2).

**DISCUSSION**

The present study showed the following results: i) compared to healthy control subjects, patients with hyperhomocysteine-
Homocysteine has previously been reported to enhance LOX-1 expression in cultured bovine aortic endothelial cells (16), but the present study is, to our knowledge, the first report of elevated levels of LOX-1 mRNA in PBMC from hyperhomocysteinemic subjects. The patients also had hypercholesterolemia and had been instructed to follow the NCEP Step I diet for the previous 6 wk. The patients also had hypercholesterolemia (16), but the present study is, to our knowledge, the first report of elevated levels of LOX-1 mRNA in PBMC from hyperhomocysteinemic subjects. These data suggest that homocysteine may exert part of its atherogenic effect by enhancing LOX-1 expression, which in turn may promote oxLDL-induced responses, including TNFα release from leukocyte subpopulations. Moreover, the present study suggests that these LOX-1-related inflammatory responses may be reduced by folic acid therapy, and such effects may be beneficial in atherosclerotic patients.

Folic acid has been shown to influence the response to folic acid independent of homocysteine. In this study, however, the different polymorphisms of MTHFR were not measured.

A major finding in the present study was that folic acid supplementation reduced the LOX-1 mRNA levels as well as the oxLDL-stimulated release of TNFα from PBMC in hyperhomocysteinemic subjects. We have previously demonstrated, in the same study population, that folic acid supplementation suppresses the oxLDL-induced chemokine response in PBMC (15), and at least for one of these mediators (i.e., monocyte chemoattractant protein-1), LOX-1 seems to be of major importance for the oxLDL-induced response (19). Thus, our findings in this and previous studies (15) suggest that folic acid therapy may suppress the inflammatory response to oxLDL in PBMC from patients with hyperhomocysteinemia, and this anti-inflammatory effect could at least partly involve LOX-1-related mechanisms. Although the reduction in the LOX-1 mRNA levels as well as in the oxLDL-stimulated release of TNFα from PBMC during folic acid treatment occurred in parallel with a marked decrease in plasma homocysteine levels, anti-inflammatory effects of folic acid independent of homocysteine-lowering effects cannot be excluded. Nevertheless, folic acid treatment may be an effective, inexpensive therapeutic approach to reduce inflammation in hyperhomocysteinemic patients, and if this enhancement of LOX-1 expression and secretion of inflammatory cytokines in hyperhomocysteinemia is atherogenic, the inhibition of mononuclear cells within the vessel wall, such anti-inflammatory effects could also reduce atherogenesis in these patients.

The present study suggests that elevated levels of LOX-1 and enhanced oxLDL-induced TNFα responses in cells involved in atherogenesis may be among the pathophysiological consequences of hyperhomocysteinemia. Moreover, our findings suggest that folic acid supplementation may downregulate these inappropriate responses in these patients.

**LITERATURE CITED**


