Circulating Lipid Hydroperoxide Levels in Human Hyperhomocysteinemia
Relevance to Development of Arteriosclerosis

N.P.B. Dudman, D.E.L. Wilcken, and R. Stocker

Elevated circulating homocyst(e)ine is a risk factor for occlusive vascular disease. We explored whether elevated plasma homocyst(e)ine is associated with increased plasma lipid hydroperoxides that might trigger vascular disease. We obtained plasma containing high levels of homocyst(e)ine from four patients with a homozygous deficiency of cystathionine β-synthase activity and also from four heterozygotes with a deficiency of this enzyme after an oral methionine load. The mean plasma non-protein-bound homocyst(e)ine level in all subjects was more than 11-fold higher than the mean normal fasting value. Levels of high density lipoprotein (HDL) cholesteryl ester hydroperoxides (CEOOH), normalized against the concentration of free cholesterol in HDL, were not elevated in our subjects (mean±SD, 0.0091±0.0061) compared with values for 14 fasting healthy donors (0.0164±0.0086). An inverse dependency was observed between plasma total homocyst(e)ine and HDL CEOOH ($r=-0.78$, $p=0.023$). Also, the ubiquinol-10/ubiquinone-10 ratio in HDL, which is expected to fall during oxidative stress, increased with plasma homocyst(e)ine. Since HDL contains the majority of detectable plasma lipid hydroperoxides, of which CEOOHs are the most abundant, our data suggest that an elevated plasma homocyst(e)ine level does not enhance oxidative stress, increase the levels of lipid hydroperoxides in plasma, or generate vascular damage by this mechanism. (Arteriosclerosis and Thrombosis 1993;13:512-516)

KEY WORDS • plasma homocyst(e)ine • oxidative stress • cystathionine β-synthase • ubiquinol • cholesteryl ester hydroperoxides • HDL

The accumulation of lipid in the vessel wall plays a key role in the development of atherosclerosis. In vitro experiments show that lipid uptake by cell types involved in atherosclerotic lesions, including macrophages and smooth muscle cells, is slow unless it is presented in a chemically or physically modified form. Thus, these cell types take up acetylated and oxidized lipids more readily than unmodified lipid. In a search for possible mechanisms to explain homocyst(e)ine-induced thrombosis and the development of arteriosclerosis, the possibility that homocyst(e)ine could catalyze the in vivo peroxidation of serum lipids has been raised. It was envisaged that elevated circulating levels of homocyst(e)ine could stimulate the uptake of lipids by subendothelial tissue, leading to the formation of atherosclerotic plaque. The proposition that homocyst(e)ine could cause peroxidation of lipid is supported by the well-known ability of aliphatic sulfhydryl compounds, such as homocysteine, to reduce atmospheric oxygen to hydrogen peroxide in vitro. This reaction is catalyzed by transition metal ions, such as cupric and ferric ions, and involves the generation of free-radical intermediates.

We reasoned that if homocyst(e)ine-dependent production of free radicals and hydrogen peroxide occurred, this could conceivably lead to increased plasma levels of lipid hydroperoxides in persons with elevated plasma homocyst(e)ine. In the present study, we tested this possibility by assessing lipid hydroperoxide levels in subjects with elevated plasma homocyst(e)ine, including patients with homocystinuria due to homozygous deficiency of cystathionine β-synthase activity, and in subjects heterozygous for cystathionine β-synthase deficiency during an oral methionine load. To measure lipid hydroperoxides, we used the highly sensitive and selective high-performance liquid chromatography (HPLC) postcolumn chemiluminescence method. Since high density lipoprotein (HDL) contains the majority of plasma lipid hydroperoxides, of which cholesteryl ester hydroperoxides (CEOOHs) are the most abundant, our study has focused on plasma HDL CEOOH levels. In addition, we have assessed in these subjects the ratio of plasma ubiquinol-10 ($\text{CoQ}_{10}\cdot\text{H}_2$) to ubiquinone-10 ($\text{CoQ}_{10}$), which is thought to reflect the level of oxidative stress in the plasma and its main lipoprotein fractions.

Methods

Four patients, aged 20–45 years, with homocystinuria due to homozygous deficiency of cystathionine β-syn-
those activity were investigated. Each patient was currently on a therapy regimen that included pyridoxine (100 mg daily) and folic acid (5 mg daily). Three of the patients were also taking betaine (3 g twice daily), although this was not taken on the morning of the test. All four patients had been poor responders to pyridoxine therapy.

Four obligatory heterozygotes for cystathionine β-synthase deficiency activity, aged 43–81 years, were also studied. These subjects had each parented at least one child with homocystinuria and, during prior oral methionine load tests, were found to have plasma homocyst(e)ine levels elevated substantially above the appropriate normal mean ± 2 SD. Healthy adult control subjects for lipid analyses comprised eight men and six women, aged 25–36 years.

Three patients with homocystinuria underwent a 10-hour overnight fast and then provided a venous blood sample for lipid and amino acid analysis. The fourth patient, who in addition had diabetes mellitus and was taking betaine, ate a small breakfast low in protein and fat before providing a blood sample. The heterozygotes, after fasting for at least 9 hours, took an oral load of L-methionine (4 g/m² body surface area) in 100 mL apple juice to boost their plasma homocyst(e)ine levels. After a further 6 hours, these subjects provided blood for analysis of lipids and amino acids. These procedures were performed with the informed consent of all subjects and in accordance with institutional guidelines.

Blood for lipid and amino acid analysis was collected into lithium-heparin tubes and centrifuged immediately. For amino acid analysis only, plasma was separated, its protein was precipitated using sulfosalicylic acid containing γ-aminoxybutyric acid as an internal standard, and the protein-free supernatant was analyzed for amino acid content, as previously described, with a Beckman 6300 amino acid analyzer fitted with a 10-cm cation-exchange column (type 339051) and eluted with lithium citrate buffers. Plasma homocyst(e)ine, when determined by this process, is oxidized and measured as the disulfides cysteine-homocysteine and homocystine. The total concentration of these non-protein-bound forms of homocyst(e)ine is expressed as the sum of the molar concentration of cysteine-homocysteine mixed disulfide plus twice the concentration of homocystine.

Fasting plasma for the analysis of lipids was rapidly processed; the same time elapsed between blood collection and analysis for every sample. HDL and low density lipoprotein (LDL) were isolated by high-speed centrifugation using a scaled-down published method. Lipids in fresh fasted plasma and in the HDL and LDL fractions were extracted by adding 0.2 mL plasma, HDL, or LDL to 2.0 mL methanol and 10 mL n-hexane (4°C) for 1 minute. The residue from the evaporated upper phase was redissolved in 200 μL ethanol for analysis of unoxidized lipids and lipid hydroperoxides or in 2.0 mL Beckman lithium-S buffer for amino acid analysis. Lipids were separated by HPLC on a C-18 column, and the effluent was monitored at 210 nm for cholesterol and other unoxidized lipids before being mixed with a microperoxidase-isoluminol solution to measure lipid hydroperoxides by chemiluminescence. Concentrations of CoQ10 and CoQ9 were determined by HPLC with electrochemical detection. The rate of reduction of ubiquinone-1 (CoQ1), 100 μmol/L by homocysteine (100 μmol/L) in sodium phosphate buffer (100 mmol/L, pH 7.4, containing 0.154 mol/L NaCl) at 25°C was monitored at 280 nm. CoQ1 was used here rather than CoQ9 because of the greater aqueous solubility of CoQ1.

**Results**

The patients with homocystinuria, as expected, had elevated fasting plasma homocyst(e)ine levels of 59.7 ± 22.0 (mean ± SD) μmol/L (Table 1) compared with normal levels of 4.2 ± 0.8 from control subjects (n = 24) (see Reference 12). The levels of HDL CEOOH in these patients were not elevated and, in fact, were all below the normal mean (Table 1). Plasma CEOOH levels were also below the normal mean (not shown). Values of the CoQ10/H2/CoQ10 ratio in these patients were significantly elevated above the normal mean in HDL, LDL, and whole plasma. In HDL and whole plasma, the mean values of the ratio were above the normal mean ± 2 SD, and in LDL, the mean value for patients was above the normal mean + SD.

All heterozygotes for deficiency of cystathionine β-synthase, after an oral methionine load, also had plasma homocyst(e)ine levels above their respective normal mean ± 2 SD for a 4-hour plasma sample during an oral methionine load (Table 1). All levels of HDL CEOOH for this group fell within the normal range defined by the mean ± 2 SD. These subjects also had normal or elevated values for the CoQ10/H2/CoQ10 ratio for their HDL fraction and values that fell within the range (mean ± 2 SD) for LDL. Ratios for whole plasma in two subjects were slightly above the normal mean ± 2 SD.

There was a clear inverse dependence between HDL CEOOH and plasma homocyst(e)ine in our eight plasma samples (Figure 1; r = −0.78, p = 0.023). In a parallel finding, the CoQ10/H2/CoQ9 ratio increased with the concentration of plasma homocyst(e)ine (Figure 2), while absolute levels of CoQ10 remained normal (data not shown). No significant correlation occurred between the age of the subject and plasma homocyst(e)ine, plasma HDL CEOOH levels, or values of the plasma HDL CoQ10/H2/CoQ9 ratio in our eight subjects. Control measurements revealed no detectable homocysteine, homocysteine, or homocysteine-cysteine mixed disulfide in the organic upper-phase extracts of plasma, even when additional homocysteine at a final concentration of up to 100 μmol/L had been added to plasma before extraction. Likewise, neither cysteine, cystine, nor homocysteine-cysteine mixed disulfide was extracted, even from plasma spiked with cysteine up to 100 μmol/L. We found that reduction of CoQ1 by homocysteine was negligible (<1%) after 40 minutes at 25°C.

**Discussion**

In studying the possibility of oxidative stress in homocysteinemic subjects, we have focused on plasma CEOOH and the CoQ10/H2/CoQ10 ratio for the following reasons. Whereas lipid hydroperoxides are not the final product of lipid oxidation, this is also true for other products that are measured as an index of lipid peroxidation, including malondialdehyde (MDA) equivalents in the thiobarbituric acid–reactive substances (TBARS) assay. In contrast to MDA, CEOOHs in HDL and LDL...
TABLE 1. Plasma HDL Cholesteryl Ester Hydroperoxides, Ratio of Ubiquinol-10/Ubiquinone-10 in Plasma Lipoprotein Fractions, and Plasma Non–Protein-Bound Homocyst(e)ine in Subjects With Elevated Plasma Homocyst(e)ine Levels

<table>
<thead>
<tr>
<th>Patient (sex)</th>
<th>HDL CEOOH/free cholesterol (×10^-2)</th>
<th>CoQ_{H_2}/CoQ_{10}</th>
<th>Plasma homocyst(e)ine (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homocystinuria patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (F)</td>
<td>14.1</td>
<td>8.8</td>
<td>6.3</td>
</tr>
<tr>
<td>2 (M)</td>
<td>10.9</td>
<td>6.5</td>
<td>7.1</td>
</tr>
<tr>
<td>3 (M)</td>
<td>Not detected</td>
<td>10.1</td>
<td>8.7</td>
</tr>
<tr>
<td>4 (M)</td>
<td>4.5</td>
<td>9.6</td>
<td>10.4</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 (F)</td>
<td>5.5</td>
<td>9.0</td>
<td>7.1</td>
</tr>
<tr>
<td>6 (M)</td>
<td>6.7</td>
<td>7.1</td>
<td>4.9</td>
</tr>
<tr>
<td>7 (F)</td>
<td>11.9</td>
<td>5.6</td>
<td>0.5</td>
</tr>
<tr>
<td>8 (F)</td>
<td>19.1</td>
<td>1.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Control subjects (n = 14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SD‡</td>
<td>16.4±8.6</td>
<td>1.6±1.3</td>
<td>4.3±2.6</td>
</tr>
</tbody>
</table>

HDL, high density lipoprotein; HDL CEOOH, HDL cholesteryl ester hydroperoxides (normalized against the free cholesterol concentration); CoQ_{H_2}/CoQ_{10}, ubiquinol-10/ubiquinone-10; LDL, low density lipoprotein; F, female; M, male.

*The mean±SD for fasting plasma homocyst(e)ine from all healthy control subjects (n=24) is 4.2±0.8 μmol/L (see Reference 12).
†Our values for the mean+2 SD plasma homocyst(e)ine show that there is little change at 4 and 8 hours after methionine load: for 11 normal postmenopausal women (compare with patient 5), 22.4 and 24.6 μmol/L, respectively; for 21 normal men (compare with patient 6), 16.2 and 17.4, respectively; and for 25 normal premenopausal women (compare with patients 7 and 8), 14.8 and 17.4, respectively. For this reason, we have compared patients' plasma homocysteine 6-hour postmethionine levels directly with normal values.
‡See Reference 6.

are remarkably stable in human plasma, with no significant decrease for the first 24 hours at 37°C. In relation to the CoQ_{H_2}/CoQ_{10} ratio in the peripheral circulation, in humans this appears to be independent of the total amount of circulating coenzyme Q (CoQ). To date, there are no known enzymatic processes in plasma that affect this ratio. Under all oxidizing conditions that we have studied in vitro, (plasma) lipoprotein-associated CoQH_2 is the first lipid-soluble antioxidant to be consumed, becoming oxidized and stoichiometrically converted to CoQ. Where examined, this also holds true in tissues and in vivo situations of oxidative stress. Circulating levels of lipid hydroperoxides inversely correlate with the HDL CoQH_2/CoQ ratio. For these reasons, determinations of CEOOH, together with assessment of the CoQ_{H_2}/CoQ_{10} ratio, seem to be valid measures for oxidative stress in vivo.
The results of the present study clearly show that elevated plasma homocyst(e)ine does not lead to increased endogenous levels of plasma HDL CEOOH. This is emphasized by the following findings. First, our subjects, all of whom had substantially elevated plasma homocyst(e)ine levels, all had normal or lower-than-normal plasma levels of HDL CEOOH. Second, and somewhat unexpectedly, plasma HDL CEOOH levels were inversely proportional to the concentration of plasma homocyst(e)ine (Figure 1), with a probability of \( p = 0.023 \). Third, this is supported by the positive relation between values of the indicator of oxidative stress, the \( \text{CoQ}_{10}/\text{CoQ}_{0} \) ratio, and the concentration of plasma homocyst(e)ine (Figure 2). Fourth, subjects with the higher plasma homocyst(e)ine levels also had higher-than-average levels of the \( \text{CoQ}_{10}/\text{CoQ}_{0} \) ratio, indicating that elevated plasma homocyst(e)ine do not lead to increased levels of oxidized plasma lipids and antioxidants.

Although the ages of our healthy control subjects for lipid analysis do not range as high as the ages of our subjects with homocysteinemia, this seems unlikely to have altered the outcome of our study, because the extent of oxidation of biomolecules, including lipids, is generally thought to increase with age. By contrast, we found that plasma lipids in our homocysteinemic subjects were actually more reduced than plasma lipids from our younger control group. The inverse link between ex vivo plasma homocyst(e)ine and lipid hydroperoxide concentrations is novel. It contrasts with the in vitro formation of lipid peroxides during aerobic coincubation of lipid with homocysteine in Ham's F-10 medium, which was catalyzed by cultured aortic endothelial cells. These contrasting results may reflect the substantial concentrations of free iron and copper ions in Ham's F-10 medium, which are not present in human plasma but would be expected to enhance catalysis of homocysteine autoxidation in the F-10 medium. Whether increased levels of plasma homocyst(e)ine actually give rise in vivo to increased plasma concentrations of low-molecular-weight thiol compounds is not yet clear. However, the inverse link may well be related to our finding that values of the \( \text{CoQ}_{10}/\text{CoQ}_{0} \) ratio in HDL increased with plasma homocyst(e)ine. Although the mechanisms of these two phenomena are currently obscure, they do not appear to be related to the age of the subjects who have elevated plasma homocyst(e)ine. One possibility is that increased plasma homocyst(e)ine causes the \( \text{CoQ}_{10}/\text{CoQ}_{0} \) ratio to rise, which would then provide greater protection of lipoprotein lipids against hydroperoxidation. Such an increase in the \( \text{CoQ}_{10}/\text{CoQ}_{0} \) ratio would be unlikely to result from uncatalyzed reduction of \( \text{CoQ}_{0} \) by homocysteine, on the basis of our control experiment.

The concentrations of plasma homocyst(e)ine recorded here, ranging from 26.5 to 92.2 \( \mu \text{mol/L} \), are lower than would be expected in severe untreated homocystinuria due to cystathionine \( \beta \)-synthase deficiency. However, they are of the same order as, or considerably higher than, levels found in patients with premature occlusive vascular disease and impaired homocysteine catabolism, whose circulating homocyst(e)ine is thought to contribute to their premature vascular disease. Thus, the possibility that the vascular lesions of such patients are induced by homocysteine-induced production of lipid hydroperoxides and accelerated uptake of these oxidized products by subendothelial tissues seems improbable. This view is supported by the finding in our laboratory (data not shown) and elsewhere that arteriosclerotic lesions in young patients with homocystinuria may contain little or no evidence of accumulated lipid in foam cells or extracellularly.

The hypothesis that homocysteinemia leads to increased lipid peroxidation has also been studied recently by Blom et al. They likewise concluded that high serum homocysteine concentrations were not associated with increased lipid peroxidation products in serum. Blom et al used two indirect methods to assess plasma lipid hydroperoxide concentrations that were based on the measurement of 1) protein-associated fluorescent products, after removal of lipids by solvent extraction, and 2) MDA in serum by the TBARS method. Results obtained from these two assays are not directly comparable with our results because they do not measure lipid hydroperoxides as such, in contrast to our HPLC procedure with chemiluminescence detection.

The results of Blom et al for individual subjects vary by up to 40-fold between the two assay procedures, assuming a serum protein concentration of 60 g/L. For this reason and because the levels of analytes measured by Blom et al using the two assays are two to four orders of magnitude higher than the concentration of lipid hydroperoxides measured in fresh plasma or serum by HPLC-chemiluminescence, caution is indicated in applying the results obtained with these two assays to the actual level of lipid hydroperoxides in fresh plasma or serum. The TBARS assay, although used widely, is known to be affected by many potential artifacts, particularly when applied to complex biological samples such as serum. For this reason, it is possible that most of the "reactive material" measured, at least in the TBARS assay, could have been produced during sample preparation and was not present in the freshly prepared serum.

Our observations emphasize that the thrombosis and premature arteriosclerosis that are found in homocystinuria appear to be unrelated to lipid hydroperoxidation. Thus, homocystinuria due to deficiency of cystathionine \( \beta \)-synthase could represent a nonlipid model for the initiation of arteriosclerosis.

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