Plasma Homocysteine Is Weakly Correlated with Plasma Endothelin and von Willebrand Factor but not with Endothelium-dependent Vasodilatation in Healthy Postmenopausal Women

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Background: Hyperhomocysteinemia is an independent cardiovascular risk factor, possibly through the induction of endothelial dysfunction. The postmenopausal state is associated with increased plasma homocysteine. We examined whether increased homocysteine is associated with impaired endothelial function.

Methods: Sixty-three hysterectomized but otherwise healthy postmenopausal women (54.8 ± 3.5 years) participated in this study. Fasting total plasma homocysteine (tHcy) was measured as free plus protein-bound homocysteine. Endothelial function was assessed by measuring plasma concentrations of the endothelium-derived proteins endothelin (ET), von Willebrand factor (vWF), and plasminogen activator inhibitor type 1 (PAI-1) as well as brachial artery flow-mediated, endothelium-dependent vasodilatation (FMD).

Results: Plasma tHcy was 9.6 ± 2.5 μmol/L. After adjustment for possible confounders, a 1 μmol/L increase in tHcy was associated with an increase in ET of 0.08 ng/L (P = 0.045) and an increase in vWF of 4.2% (P = 0.05). No statistically significant association was present between tHcy and PAI-1 or FMD.

Conclusions: Increased fasting homocysteine in postmenopausal women may impair some aspects of endothelial function. It is of clinical interest to study whether homocysteine lowering can improve endothelial function and thus cardiovascular morbidity and mortality in postmenopausal women.

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Hyperhomocysteinemia is an independent risk factor for atherosclerosis and thromboembolic disease. These associations are not limited to severe hyperhomocysteinemia, such as that related to homozygous cystathionine-β-synthase deficiency; they are also observed when fasting or postmethionine concentrations are moderately increased (1–3). Increased total homocysteine (tHcy) is thought to confer a graded risk of atherothrombotic disease and may be attributed to commonly occurring genetic and acquired factors such as the C677T mutation in the methylenetetrahydrofolate reductase gene, impaired renal function, inadequate folic acid and/or vitamin B6 and B12 intake, and the use of certain medications. Sex steroids also affect the tHcy concentration (4). The tHcy concentration is higher in men than in women (5, 6). Low tHcy concentrations are present during pregnancy, a state characterized by high concentrations of endogenous estrogens (7), as well as in premenopausal compared with postmenopausal women (8). In addition, in postmenopausal women, administration of hormone replacement therapy has been reported to lower homocysteine (9–11). It may, therefore, be hypothesized that a menopause-associated increase in homocysteine contributes to the postmenopausal increase in cardiovascular risk.
Homocysteine may promote atherothrombogenesis by a variety of mechanisms. Homocysteine affects the balance between coagulation and fibrinolysis, in which the endothelium plays a key role (12). It adversely affects the production of endothelium-derived nitric oxide, and it induces proliferation of vascular smooth muscle cells (12–14). However, it is not known whether tHcy modulates endothelial function in postmenopausal women. We therefore examined, in a population of healthy postmenopausal women, whether an increased tHcy concentration was associated with impaired endothelial function. As estimates of endothelial function, we assessed the endothelium-derived regulatory proteins, endothelin (ET), von Willebrand factor (vWF), and plasminogen activator inhibitor type 1 (PAI-1) as well as the brachial artery flow-mediated, endothelium-dependent vasodilatation (FMD). High plasma concentrations of ET, vWF, and PAI-1 are associated with a poor cardiovascular outcome (15–20).

**Subjects and Methods**

We recruited 68 healthy, postmenopausal women, ages 40–60 years. All subjects had undergone a hysterectomy and were screened for a long-term follow-up study on estrogen monotherapy. Hysterectomy had been performed because of a benign endometrial abnormality (n = 54), uterine prolapse (n = 9), and/or a benign ovarian abnormality (n = 5). Postmenopausal status was defined as serum estradiol ≤73 pmol/L and follicle-stimulating hormone (FSH) ≥40 IU/L. Exclusion criteria were a history of breast carcinoma and/or recent thromboembolism, evidence of liver disease (serum bilirubin >34 μmol/L, alanine aminotransferase >100 U/L, and/or alkaline phosphatase >300 U/L) and/or renal dysfunction (serum creatinine >180 μmol/L), diabetes mellitus requiring pharmacological therapy, use of lipid-lowering drugs and/or anticonvulsives, and use of sex steroids and/or corticosteroids more recently than 6 months before the start of the study. Five subjects were subsequently excluded because of lack of sufficient plasma for tHcy measurement (n = 4) or because of rheumatoid arthritis (n = 1). All subjects gave informed consent, and the protocol was approved by the local ethics committee. Subject characteristics are summarized in Table 1.

**GENERAL PROCEDURES**

In all subjects, we assessed current smoking status (yes/no), alcohol use (units per week), and blood pressure. Blood pressure (mean of four readings) was measured on the left arm after 10 min of rest, using an automated device (BP-8800; Colin). Hypertension was defined as a systolic blood pressure ≥160 mmHg and/or a diastolic blood pressure ≥95 mmHg and/or the use of antihypertensive drugs. As indexes of body composition, we assessed the body mass index (BMI; kg/m²) and the waist-to-hip ratio (WHR; waist circumference divided by hip circumference). Blood samples were collected between 0830 and 1130 in the morning after an overnight fast. Plasma and serum samples, prepared from the blood samples, were then either used directly (to determine serum estradiol, FSH, lipids, and glucose) or stored at −70 °C until analysis (for the determination of serum insulin and plasma tHcy, ET, vWF, PAI-1, and C-reactive protein).

**tHcy and Endothelium-Derived Regulatory Proteins**

Plasma concentrations of tHcy, ET, vWF, and PAI-1 were assayed in a single run. Fasting plasma tHcy was measured as free plus protein-bound homocysteine by HPLC with fluorescence detection (21). The intraassay CV was 2%; the lower limit of detection was 0.5 μmol/L. Plasma immunoreactive ET was measured by RIA (Nichols Institute) after extraction on Sep-Pak C₁₈ cartridges (Waters). The intraassay CV was 10%. The lower limit of detection of the assay, defined as the lowest single value that could be distinguished from zero at the 99% confidence limit, was 2 ng/L. Cross-reactivity with ET-1 was 100%, with ET-2 was 67%, with ET-3 was 84%, and with “big” ET-1 was 2.6%. vWF antigen was measured by a peroxidase ELISA (22), using rabbit anti-vWF antigen IgG in the coating buffer and peroxidase-conjugated rabbit anti-vWF antigen IgG (Dako). o-Phenylenediamine (Sigma Chemical Co.) was used as substrate. The intraassay CV was 2.3%. vWF concentrations were expressed as percentages of normal pooled plasma, the antigenic concentration of which is defined as 100%. We used a carefully standardized procedure (23) for samples to be used for determination of the antigenic concentrations of PAI-1. The plasma PAI-1 concentration was determined by ELISA (Innotest PAI-1; Innogenetics). The intraassay CV was <10%; the detection limit was 2.5 μg/L.

**Other Laboratory Variables**

Serum estradiol was measured using a RIA (Double Antibody; Diagnostic Products). The intra- and interassay CVs were both 5.9%; the lower limit of detection was 5 pmol/L. Serum FSH was measured using a microparticle enzyme immunoassay (IMx; Abbott Laboratories). The intra- and interassay CVs were 2.4% and 9.6%, respectively. Serum total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured with enzymatic methods (Boehringer Mannheim). LDL-cholesterol was calculated by the Friedewald formula. Serum glucose was measured by a routine method. Finally, the serum insulin and C-reactive protein plasma concentrations were measured, both assayed in a single analytical run. The serum insulin was measured by a RIA (Biosource Diagnostics). The intraassay CV was 5%. The C-reactive protein plasma concentration was measured using an in-house ELISA, with rabbit anti-CRP (Dako) as the capture and tagging antibodies. The intraassay CV was <10%; the detection limit was 0.01 mg/L.
**ENDOTHELIUM-(IN)DEPENDENT VASODILATATION**

Measurements of FMD were performed after the subjects had refrained from smoking and from the use of caffeine-containing beverages for at least 4 h. We used a vessel wall movement detector system (Wall Track System; Neurodata), which consists of an ultrasound imager (Ultramark IV; ATL) connected to a data acquisition and processing unit. In M-mode (motion-mode), in which the vessel wall distension is repeatedly registered during a period of 5–6 s, the arterial diameter can be obtained with an accuracy of 0.1–0.2 mm. The baseline end-diastolic brachial artery diameter was measured after 15 min of rest in the supine position. Reactive hyperemia was induced by the release of a blood pressure tourniquet that had been inflated around the forearm for 4 min at a pressure of 100 mmHg above the systolic blood pressure. The brachial artery diameter was again measured between 45 and 60 s after the release of the cuff. After a period of 15 min, a second baseline measurement was performed. Endothelium-independent vasodilatation was measured 5 min after sublingual administration of 0.4 mg of nitroglycerin (NTG). Endothelium-(in)dependent vasodilatation was expressed as the percentage of increase of the arterial diameter from baseline. In our hands, the CVs for the measurements of baseline diameter and endothelium-dependent and -independent vasodilatation were 4.6%, 5.5%, and 7.7%, respectively (24).

**STATISTICAL ANALYSIS**

Data are presented as mean (SD) and range. In cases of skewed distribution, the geometric mean is presented. Pearson correlation analysis was used to describe the relationship between endothelium-(in)dependent vasodilatation and endothelium-derived regulatory proteins on the one hand and tHcy on the other. To study whether tHcy was independently associated with endothelial function parameters, we constructed univariate and multivariate models. Univariate analyses were used to study the relationship between the dependent variables (ET, vWF, PAI-1, FMD, and NTG-induced vasodilatation) and possible determinants thereof: tHcy, age, serum estradiol and FSH concentrations, smoking habits, alcohol use, variables of the insulin resistance syndrome (plasma concentrations of glucose and insulin, serum lipids, blood pressure, BMI, and WHR), and as an estimate of low-grade inflammatory state, the C-reactive protein concentration. For variables with a skewed distribution, the logarithmically transformed data were used. Variables that had a P value <0.15 in the univariate analyses were entered into a multivariate model. The residuals were checked for gaussian distribution and constancy of variance. All testing was two-tailed with 0.05 as the level of significance.

**Results**

The characteristics of the participants are shown in Table 1. The mean tHcy plasma concentration was 9.6 ± 2.5 μmol/L. Ten subjects (16%) had a tHcy concentration ≥12 μmol/L, the cutoff value used in the European Concerted Action Project (1) to define an increased fasting tHcy concentration.

As Fig. 1 shows, tHcy was associated with ET (r = 0.25; P = 0.04) and tended to be associated with vWF (r = 0.22; P = 0.08) but was not associated with PAI-1, FMD, or NTG-induced vasodilatation (r = 0.17, −0.10, and −0.05, respectively). ET was associated with vWF (r = 0.30; P = 0.02); neither ET nor vWF were associated with PAI-1; FMD tended to be associated with NTG-induced vasodilatation (r = 0.23; P = 0.07). No association existed between the endothelium-derived regulatory proteins and endothelium-(in)dependent vasodilatation.

Adjustment for possible confounders did not affect the association between the tHcy and ET plasma concentrations nor between the tHcy and vWF plasma concentrations (Table 2A). A 5 μmol/L increase in tHcy was thus associated with an increase in ET of 0.4 ng/L (95% confidence interval, 0.01–0.85) and an increase in vWF of 20.8% (95% confidence interval, −0.2% to 41.8%).

PAI-1 was significantly associated with the BMI and WHR (both r = 0.49) and with the concentrations of insulin (r = 0.49), glucose (r = 0.26), serum total cholesterol (r = 0.43), triglycerides (r = 0.54), estradiol (r = 0.31),
and FSH (r = 0.26), and C-reactive protein (r = 0.31) but not with tHcy. Substitution of the tissue-type plasminogen activator antigen concentration or the tissue-type plasminogen activator/PAI-1 ratio for the PAI-1 concentrations gave very similar results (data not shown).

**Discussion**

This is the first study that evaluates the association between increased tHcy and endothelial function parameters in healthy postmenopausal women. We found an independent, although rather weak, association between fasting plasma tHcy and ET as well as between fasting tHcy and vWF. A 5 μmol/L increase in plasma tHcy (1 SD in most populations) was associated with an increase of ~15–20% of these potential markers of endothelial dysfunction, which may not be trivial. No association existed between the fasting tHcy concentration on the one hand and the PAI-1 concentration or endothelium-dependent vasodilatation on the other hand.

Experimental evidence suggests that the propensity toward atherothrombosis associated with hyperhomocysteinemia results from endothelial dysfunction and injury followed by platelet activation and thrombus formation (14). There are no direct methods of measuring endothelial function in humans. Estimates of different types of endothelial (dys)function, however, may be obtained indirectly by measurement of plasma concentrations of endothelium-derived regulatory proteins and endothelium-dependent vasodilatation (25). In this study, we found a significant association between tHcy and ET that remained unchanged after adjustment for other vascular risk factors. A weak but independent association existed between tHcy and vWF.

The endothelins are a family of peptides that possess characteristically sustained vasoconstrictor properties (26). ET-1 is the predominant isoform and is produced mainly by endothelial cells. vWF is also produced mainly by endothelial cells and functions as a carrier protein for factor VIII procoagulant protein; it also enhances platelet adhesion to exposed extracellular matrix. PAI-1, on the other hand, is produced not only by endothelial cells, but also by adipocytes, hepatocytes, and vascular smooth muscle cells. By complex formation with tissue-type plasminogen activator, it functions as an essential antagonist of fibrinolysis. Increased concentrations of ET, vWF, and PAI-1 are present in diseases that involve the endothelium, such as atherosclerosis (27, 28), diabetic angiopathy (25), and vasculitis (29). Moreover, prospective studies have found increased concentrations of ET, vWF, and PAI-1 to be associated with an adverse cardiovascular outcome (15–20).

Our results contrast somewhat with a recently published case-control study that found no difference in the plasma concentrations of endothelium-derived regulatory proteins between healthy subjects with and without mild hyperhomocysteinemia. In patients with peripheral arterial occlusive disease, however, that study did find higher vWF concentrations in hyperhomocysteinemic than in normohomocysteinemic subjects (30). Moreover, in hyperhomocysteinemic patients with peripheral arterial occlusive disease, 1 year of treatment with pyridoxine plus folic acid was associated not only with a decrease in tHcy but also a decrease in plasma vWF (31). Both studies were performed in young adults (<56 years) and defined hyperhomocysteinemia by an abnormal postmethionine tHcy concentration, which was not necessarily accompanied by an increased fasting tHcy concentrations. Taken together, these data and the present study suggest that increased tHcy concentrations may be a determinant of impaired endothelial function as reflected by increased ET and vWF.

We did not find any relationship between fasting tHcy
and FMD of the brachial artery. Hyperemia-induced FMD is largely NO-mediated (32, 33). Most studies to date have suggested an adverse effect of homocysteine on NO activity and/or endothelium-dependent vasodilatation (12, 13, 34–36). Whether these studies are relevant to our results is not clear. Results of in vitro and animal studies (35, 36) cannot readily be extrapolated to the human situation. The studies performed in humans investigated subjects with either substantially higher tHcy concentrations (13, 34) than in our study or, as in the study by Tawakol et al. (12), investigated elderly subjects, many of whom had additional cardiovascular risk factors. In healthy siblings of patients with premature atherosclerotic disease, Lambert et al. (37) found FMD to be inversely associated with postmethionine tHcy concentrations but not with fasting tHcy concentrations.

There is increasing evidence that homocysteine-induced vascular dysfunction is mediated through oxidative mechanisms. Autooxidation of homocysteine generates several potent reactive oxygen species that may impair endothelial function (38). Whether homocysteine-induced oxidant stress is associated with increased synthesis of ET and vWF needs further investigation. Alternatively, tHcy may reduce NO-mediated inhibition of ET generation by reducing the bioavailability of NO. The latter, however, appears less likely because we did not find any relationship between the tHcy concentration and FMD, which is largely NO-mediated.

We performed a cross-sectional analysis. Therefore, it is obvious that we must interpret the results of our study with caution. Furthermore, we assessed only the fasting and not the postmethionine homocysteine concentration. However, both the fasting and postload tHcy concentrations are strong and independent predictors of vascular disease (1).

In summary, in a well-defined group of hysterectomized, but otherwise healthy, postmenopausal women, we demonstrated an independent, although rather weak, association between the fasting tHcy concentration and the plasma ET and vWF concentrations. We did not find a relationship between tHcy and endothelium-dependent vasodilatation. Our findings suggest that during postmenopause, which is associated with a rise in tHcy, an increased fasting homocysteine concentration may impair endothelial function to some extent. It is of clinical interest to study whether lowering the homocysteine concentration can improve endothelial function and thus reduce cardiovascular morbidity and mortality in postmenopausal women.

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