Effects of Methionine-Induced Hyperhomocysteinemia on Endothelium-Dependent Vasodilation and Oxidative Status in Healthy Adults

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Background—Homocysteine-mediated endothelial dysfunction has been proposed to occur via oxidative stress mechanisms in humans. However, there is controversy regarding the effects of homocysteine on endothelial function and oxidative status, which may in part result from age discrepancy across the studies. The present study was designed to investigate the aging effect on the relationship between endothelium-dependent vasodilation and oxidative status in methionine-induced hyperhomocysteinemia.

Methods and Results—Plasma homocysteine, phosphatidylcholine hydroperoxide (PCOOH), P-selectin levels, and brachial artery flow-mediated vasodilation were measured at baseline and 4 hours after an oral methionine load (0.1 g/kg) in 15 younger (21 to 40 years) and 15 older (55 to 70 years) healthy adults. Homocysteine increased from 7.3±1.3 μmol/L at baseline to 22.7±5.2 μmol/L at 4 hours in younger (P<0.001) and from 7.4±1.4 to 24.3±4.5 μmol/L in older adults (P<0.001). PCOOH levels were not significantly different between baseline and 4 hours in both groups (P=0.10 in young; P=0.14 in old). P-selectin, which is expected to increase during oxidative stress, was not changed in older (P=0.08) but decreased in younger adults (P=0.037) at 4 hours. Flow-mediated vasodilation was preserved from 13.1±2.1% at baseline to 13.5±2.8% at 4 hours in younger (P=0.49) and decreased from 12.8±2.4% to 8.5±2.8% in older adults (P<0.001).

Conclusions—The present study demonstrates that endothelial dysfunction caused by methionine-induced hyperhomocysteinemia is age-related and is mediated through impaired nitric oxide activity without change of oxidative status. Our data do not support previous hypotheses that endothelial damage by homocysteine is via oxidative stress mechanism in humans. (Circulation. 2000;101:485-490.)

Key Words: endothelium • homocysteine • nitric oxide • vasodilation

Hyperhomocysteinemia has been known as an independent risk factor for atherosclerosis, an early manifestation of which is endothelial dysfunction. The endothelial damage caused by homocysteine has been proposed as an imbalance between nitric oxide availability and homocysteine concentrations in experimental studies. Initially, homocysteine reacts with nitric oxide to form S-nitrosohomocysteine, which preserves the bioactivity of nitric oxide with vasodilatory and antiplatelet effects. When nitric oxide formation is reduced, excessive homocysteine may further damage the endothelium by generation of reactive oxygen species, thereby increasing lipid peroxidation. In humans, impairment of endothelium-dependent flow-mediated vasodilation has recently been found in homocystinuric children and in older healthy adults with moderate fasting hyperhomocysteinemia. The key abnormality of the endothelium is impaired release of nitric oxide in response to flow. More recently, studies have also shown that flow-mediated vasodilation is impaired in healthy adults with methionine-induced mild hyperhomocysteinemia. This finding demonstrates the direct relationship between changes of homocysteine concentrations and endothelial function, and this is informative because mild hyperhomocysteinemia (15 to 30 μmol/L) is common in the population (normal range, 5 to 15 μmol/L) associated with genetic defects of enzymes involved in homocysteine metabolism, vitamin (folate, B12, or B6) deficiency, or disease states such as renal failure. The adverse effects of such mild hyperhomocysteinemia on the endothelium have been proposed to occur via oxidative stress mechanisms because endothelium-dependent vasodilation is ameliorated after administration of folic acid and vitamin C, both of which are able to scavenge superoxide anion. However, conflicting results have been found in young healthy adults, who have no change in endothelium-dependent vasodilation or oxidative status after methionine load. Aging is associated with progressive decrease of nitric
oxide and increase of peroxidative stress; the effects of mild hyperhomocysteinemia on endothelium-dependent vasodilation and oxidative status are age-related remains less clear. To clarify the aging effect, we investigated the effects of methionine-induced hyperhomocysteinemia on the relationship between endothelium-dependent flow-mediated vasodilation and oxidative status in different age groups.

Methods

Subjects and Study Design

To consider the effect of aging, we prospectively defined 2 age groups for inclusion in this study. The younger adults were defined as those between 21 and 40 years of age and the older adults as those between 55 and 70 years of age. We recruited 30 healthy Chinese adults (4 men and 26 women, aged 21 to 70 years) from hospital staff and community volunteers. Subjects were included only if they were clinically healthy, nonsmokers, and had no hypertension, diabetes mellitus, hyperlipidemia, or history of premature vascular disease. No subjects were taking regular medications. All subjects gave written informed consent and the study was approved by the local ethics committee.

After an overnight fast (10 to 14 hours), venous blood samples were drawn from all volunteers to measure the concentrations of homocysteine, phosphatidylcholine hydroperoxide (PCOOH), P-selectin, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, glucose, folate, and vitamin B12 levels. Supine blood pressure was measured for all subjects and 10 minutes later all sound measurements were performed according to the method described by Celermajer et al, using a high-resolution ultrasound machine (Advanced Technology Laboratories 3000 System) equipped with an L10-5 linear array transducer. Arterial diameters were measured at rest, during reactive hyperemia, again at rest (after vessel recovery), and after administration of 0.6 mg sublingual nitroglycerin. The condition of reactive hyperemia was induced by inflation of a pneumatic cuff on the upper arm to suprasystolic pressure, followed by cuff deflation after 4.5 minutes. The brachial artery was scanned in longitudinal section 2 to 8 cm above the elbow, and the arterial diameter was measured on B-mode images with the use of ultrasonic calipers. The end-diastolic arterial diameter was measured from one media-adventitia interface to the other at the closest section 3 times at baseline, every 30 seconds after reactive hyperemia, and after administration of nitroglycerin. The maximum vessel diameter was taken as the average of the 3 consecutive maximum diameter measurements after hyperemia and nitroglycerin, respectively. Vasodilation was then calculated as the percent change in diameter compared with baseline. In our laboratory, all measurements were performed by 2 independent investigators. The intraobserver and interobserver variations were 1.6% and 2.6%, respectively.

Laboratory Assays

Venous blood samples were placed into tubes containing EDTA. Samples were centrifuged within 30 minutes at 2000 rpm for 10 minutes. The plasma was then separated and stored at –70°C until analysis. Total homocysteine concentrations were measured by fluorescence polarization immunoassay (Abbott IMX System), which correlates well with high performance liquid chromatography. The coefficients of variation were within 5.2%. PCOOH concentrations were determined in duplicate by chemiluminescence-high performance liquid chromatography, which is very advantageous because of its specificity and sensitivity to hydroperoxides. Briefly, the total lipids were extracted from 0.5 mL of plasma, with 2 mL of chloroform and methanol mixture (2:1, vol/vol, containing 0.002% butylated hydroxytoluene as an antioxidant) added, followed by vigorous mixing. The extraction was repeated and the chloroform layer collected. After dehydration and evaporation of the combined chloroform layer, the dried total lipid residue was diluted with 40 µL of chloroform-methanol (2:1, vol/vol) and a 20-µL portion was used for high performance liquid chromatography. Chemiluminescence was produced through luminol oxidation during the reaction between PCOOH and cytochrome c. Plasma P-selectin levels were measured in duplicate by enzyme immunoassay specific for soluble P-selectin (R & D Systems). Folate and vitamin B12 were determined by microparticle enzyme immunoassay (Abbott AxSYM System) and lipid profiles by Eppendorf EPOS 5060 analyzer.

Vascular Studies

Endothelium-dependent flow-mediated vasodilation in response to reactive hyperemia and endothelium-independent nitroglycerin-induced vasodilation were evaluated in the brachial artery. Ultrasound measurements were performed according to the method described by Celermajer et al, using a high-resolution ultrasound

| TABLE 1. Baseline Characteristics of Younger and Older Subjects |
|------------------|------------------|------------------|
|                   | Younger Adults   | Older Adults     |   |
| Age, y            | 32±5            | 62±5             | <0.001 |
| Range             | 21–40           | 55–70            |   |
| Sex, M/F          | 2/13            | 2/13             | 1.0  |
| Height, cm        | 158±3           | 158±5            | 0.97  |
| Weight, kg        | 54.0±3.1        | 57.7±7.6         | 0.18  |
| Body mass index, kg/m² | 21.4±1.0    | 22.9±2.0         | 0.07  |
| SBP, mm Hg        | 103±6           | 104±8            | 0.65  |
| DBP, mm Hg        | 71±6            | 70±5             | 0.78  |
| Glucose, mmol/L   | 4.7±0.3         | 5.0±0.3          | 0.11  |
| Cholesterol, mmol/L | 4.8±0.6   | 5.0±0.6          | 0.61  |
| LDL               | 2.9±0.6         | 3.0±0.6          | 0.85  |
| HDL               | 1.4±0.2         | 1.5±0.2          | 0.43  |
| Triglycerides, mmol/L | 1.1±0.4   | 1.0±0.4          | 0.81  |
| Folate, µg/L      | 6.3±1.7         | 7.4±1.6          | 0.17  |
| Vitamin B12, ng/L | 476±117         | 551±107          | 0.23  |

SBP indicates systolic blood pressure; DBP, diastolic blood pressure.
load, homocysteine increased from 7.3 ± 1.3 μmol/L at baseline to 22.7 ± 5.2 μmol/L at 4 hours in the younger group (P < 0.001) and from 7.4 ± 1.4 to 24.3 ± 4.5 μmol/L in the older group (P < 0.001). Homocysteine levels were not significantly different at baseline and 4 hours between 2 groups. PCOOH levels were not significantly different between baseline and 4 hours in both groups. There were higher PCOOH levels in the older group either at baseline (103 ± 41 pmol/mL versus 60 ± 19 pmol/mL in young, P = 0.001) or at 4 hours (89 ± 24 pmol/mL versus 67 ± 20 pmol/mL in young, P = 0.002). P-selectin decreased from 49 ± 10 ng/mL at baseline to 43 ± 12 ng/mL at 4 hours in the younger group (P = 0.037). P-selectin levels were not significantly different between baseline (58 ± 10 ng/mL) and 4 hours (51 ± 17 ng/mL) in the older group (P = 0.08). P-selectin at baseline in the older group was significantly higher than that at baseline in the younger group (P = 0.019). Flow-mediated vasodilation was preserved from 13.1 ± 2.1% at baseline to 13.5 ± 2.8% at 4 hours in the younger group (P = 0.49); it decreased from 12.8 ± 2.4% at baseline to 8.5 ± 2.8% at 4 hours in the older group (P < 0.001). Flow-mediated vasodilation at 4 hours in the older group was significantly lower than that at 4 hours in the younger group (P = 0.001). Vessel size and nitroglycerin-induced vasodilation were not significantly different either between baseline and 4 hours in each group or at baseline and 4 hours between the 2 groups.

### Discussion

The principal findings of this study are (1) methionine-induced mild hyperhomocysteinemia impairs endothelium-dependent flow-mediated vasodilation mainly in older but not younger healthy adults; (2) PCOOH concentrations are not significantly changed between baseline and 4 hours after methionine load in both younger and older adults; and (3) plasma P-selectin, which is expected to increase during oxidative stress, is not significantly changed in older adults and, interestingly, is significantly decreased in younger adults at 4 hours after methionine load.

In vitro studies have shown that the endothelial response to homocysteine is dependent on the production of nitric oxide. Initial exposure of the endothelium to homocysteine leads to the formation and release of nitric oxide. In the presence of homocysteine, nitric oxide forms S-nitrosohomocysteine, which preserves the endogenous bioactivity of nitric oxide with vasodilatory and antiplatelet effects. However, in longer exposure to high concentrations of homocysteine, the adverse effects of homocysteine may result from an inability of the endothelium to support S-nitrosohomocysteine formation owing to progressive depletion of nitric oxide, which may result in impaired vasorelaxation. In humans, the effects of methionine-induced mild hyperhomocysteinemia on endothelial function are conflicting. Chambers et al and Bellamy et al showed impaired flow-mediated vasodilation at 4 hours after methionine load in 13 (aged 21 to 59 years) and 24 subjects (aged 21 to 46 years). However, Hanratty et al revealed no change in acetylcholine-mediated endothelium-dependent vasodilation in 16 young adults (aged 20 to 30 years). Although the reason for the conflicting results is not known, one possible explanation is age discrepancy across the studies; aging is associated with progressive decrease of nitric oxide, which is important in endothelial response to homocysteine. Whether the effects of such hyperhomocysteinemia on endothelial function are age-related has been less clear; therefore, we developed the present study to clarify the aging effect and found that flow-mediated vasodilation after methionine load is mainly impaired in older but not younger adults. Our findings suggest that mild hyperhomocysteinemia may reduce the nitric oxide production and thus compromise vasodilation in older adults; however, in younger adults, S-nitrosohomocysteine formation is maintained and vasodilation is preserved. Whether longer exposure of the endothelium to mild hyperhomocysteinemia leads to impaired nitric oxide bioactivity in younger adults needs further investigation. Although the endothelial response to homocysteine was intact in our younger adults, Usui et al reported that flow-mediated vasodilation was impaired after methionine-induced hyperhomocysteinemia in 10 young Japanese adults (aged 26 ± 1 years). The inconsistency is difficult to explain; whether there is ethnic or environmental disparity needs further scrutiny. In our 30 Chinese adults, the obscure difference of premethionine load endothelial dysfunction in different age groups was consistent with that of Woo et al, who have reported that older Chinese adults...
adults are less susceptible to age-related endothelial dysfunction than older white adults and proposed the environmental (dietary) advantages for the relative protection in Chinese adults.23 Our data suggest that the methionine loading test could be used as a stress test to determine age-related endothelial dysfunction in healthy Chinese adults. Elderly people are generally known to be associated with lower vitamin status.31 However, in our study, older adults had higher, though not significant, folate and vitamin B_{12} levels. A possible reason for this is that older adults in this study have a higher than average socioeconomic status,32 and may have satisfactory food intake, vitamin supplements, and thus nutritional status similar to that in younger adults.33

Cellular and animal studies have demonstrated that impaired availability of nitric oxide leaves the endothelium vulnerable to unopposed homocysteine, which generates reactive oxygen species, including superoxide anion, hydrogen peroxide, and hydroxyl radical, with consequent formation of lipid peroxidation.5–9 In humans, Usui et al have reported that coadministration of folic acid, which reduces superoxide anion generation from nitric oxide synthase and xanthine oxidase, could prevent methionine-induced endothelial dysfunction.16 Chambers et al have reported that vitamin C (as an antioxidant for superoxide anion) prevents the decrease in flow-mediated vasodilation after methionine load; they have also reported that hyperhomocysteinemia might impair endothelial function via oxidative stress mechanism.21 They have proposed that folic acid and vitamin C may attenuate degradation of nitric oxide bioactivity by decreasing oxidative stress and result in amelioration of flow-mediated vasodilation in response to homocysteine. However, these studies did not directly investigate the relation between oxidative stress and acute increases of homocysteine. Previous human studies have shown that lipid peroxidation is not increased in subjects with marked hyperhomocysteinemia.34,35 Blom et al found that LDL oxidizability and lipid peroxidation (as assessed by thiobarbituric acid reactive substances) were not increased in 10 homocysteinuric patients (aged 30±7 years) compared with age- and sex-matched healthy subjects.34,35 Dudman et al also found no increase in lipid hydroperoxides (cholesteryl ester hydroperoxides) and no reduction of ubiquinol-10/ubiquinone-10 ratio, which is thought to decrease during oxidative stress, in 4 homocysteinuric patients (aged 20 to 45 years) and 4 post-methionine load persons (aged 43 to 81 years) compared with 14 healthy adults (aged 25 to 36 years).35 Recently, Hanratty et al showed no change of endothelium-dependent vasodilation or oxidative status (as assessed by thiobarbituric acid reactive substances) after methionine load in young adults.22 However, the concomitant change of oxidative status during impairment of endothelium-dependent vasodilation, which is supposed to happen in older adults with methionine-induced hyperhomocysteinemia, has not been investigated before. Therefore, we examined the direct relationship between flow-mediated vasodilation and oxidative status after methionine load in different age groups. In this study, we evaluated oxidative status by detecting PCOOH, which is the major product of lipid peroxidation.36 Our data showed that PCOOH concentrations in older adults were higher compared with those in younger persons. Increased peroxidative stress is similarly observed in aging endothelial cells in vitro.24 However, in our study, PCOOH levels were not changed from baseline to 4 hours after methionine load in both younger and older groups. The inconsistency in lipid peroxidation between experimental and human studies may be that experimental studies have applied very high concentrations of reduced homocysteine, which generates excessive reactive oxygen species with consequent increase of lipid peroxidation. In comparison, ≈80% of homocysteine in human blood is bound to protein by disulfide linkage; the remaining homocysteine forms low molecular weight disulfides, either with itself to form the dimer or with cysteine to form the mixed disulfide.37 Only a small amount circulates as free homocysteine in reduced form, which argues against homocysteine-mediated lipid peroxidation in experimental studies. Our findings suggest that homocysteine-mediated endothelial dysfunction may result from degradation of nitric oxide, which is mediated through interaction between nitric oxide and homocysteine but not via increased oxidative stress. However, our findings do not exclude a protective role for antioxidants in hyperhomocysteinemia. Perhaps vitamin C and folic acid restore endothelial function by scavenging superoxide anion, the existence of which, however, is not related to methionine-induced hyperhomocysteinemia.

We further investigated oxidative status by measuring plasma P-selectin, which is increased during oxidative stress and is proposed as a marker of endothelial damage.38,39 P-selectin, a cell adhesion molecule, is a glycoprotein that is contained in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets.40 On activation, P-selectin is rapidly mobilized to the cell surface of endothelial cells and platelets and mediates leukocyte-endothelial cell interaction (ie, leukocyte rolling).41,42 At the same time, plasma P-selectin, a soluble form of P-selectin lacking transmembrane domain, is generated by alternative splicing of P-selectin mRNA.43 Recent studies have also demonstrated that nitric oxide can modulate P-selectin expression.44,45 In the intact circulation, inhibition of nitric oxide synthesis for 60 minutes significantly increases P-selectin translocation to the endothelial cells with enhanced leukocyte-endothelial cell interaction, which is attenuated by addition of exogenous nitric oxide.44 In the endothelial cells, inhibition of nitric oxide synthesis upregulates P-selectin mRNA expression with increased P-selectin synthesis. However, addition of exogenous nitric oxide downregulates P-selectin mRNA expression and decreases P-selectin synthesis.45 The peak effect of inhibition of nitric oxide synthesis or addition of exogenous nitric oxide occurs at 2 to 4 hours.45 Our observations showed that plasma P-selectin was not significantly changed in older adults at 4 hours after methionine load and, interestingly, it was significantly decreased in younger adults. The change of P-selectin in older adults was compatible with that of the oxidative status. However, the decrease of plasma P-selectin in younger adults was not explicable by the change of oxidative status. A possible reason is that the endothelium in younger adults has intact nitric oxide reserve and sustains nitric oxide release in response to homocysteine with formation of S-nitrosohomocysteine, which has a longer half-life.
than nitric oxide and potentiates the bioactivity of nitric oxide with consequent downregulation of P-selectin synthesis. In older adults, the release of nitric oxide with exposure to homocysteine may be initially intact but progressively decreased later, resulting in biphasic regulation of P-selectin synthesis with no significant change of plasma P-selectin levels. These findings also support the theory that mild hyperhomocysteinemia exerts its effects on the endothelium through direct interaction between homocysteine and nitric oxide but not via peroxidative activity.

In conclusion, this is the first study to elucidate the aging effect on the direct relationship between endothelium-dependent vasodilation and oxidative status in methionine-induced mild hyperhomocysteinemia. We have shown that impairment of endothelium-dependent vasodilation is age-related (mainly in older adults) and is not associated with increased oxidative status. The effects of homocysteine on the endothelium are suggested to be mediated through interaction between homocysteine and nitric oxide, but not via peroxidative activity. Our findings do not support previous hypotheses that endothelial damage by homocysteine is performed via oxidative stress mechanism in humans.

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