RAPID COMMUNICATION

Endothelial dysfunction by acute hyperhomocyst(e)inaemia: restoration by folic acid

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ABSTRACT

Recent evidence demonstrates that hyperhomocyst(e)inaemia is a novel risk factor for cardiovascular diseases. In patients with chronic hyperhomocyst(e)inaemia, endothelial function is impaired. However, whether hyperhomocyst(e)inaemia per se is a cause or an epiphenomenon of endothelial dysfunction remains unknown. In this study, we examined the effects of methionine-induced acute hyperhomocyst(e)inaemia on human endothelial function. In healthy volunteers we administered methionine (0.1 g/kg body weight, per os), a substrate of homocyst(e)ine, with or without folic acid (20 mg, per os) and examined flow-mediated vasodilatation of the brachial artery by high-resolution ultrasonography as a non-invasive measure of endothelial function. We also measured plasma levels of homocyst(e)ine before and 3, 8 and 24 h after methionine loading. Methionine administration increased plasma levels of homocyst(e)ine by four times the basal level at 8 h (P < 0.0001, ANOVA). The plasma levels returned to baseline at 24 h. Flow-mediated vasodilatation was significantly decreased to half of the baseline value at 8 h and returned to baseline at 24 h (P < 0.0001, ANOVA), whereas endothelium-independent vasodilatation by glyceryl trinitrate was not affected by the methionine loading. Co-administration of folic acid did not attenuate methionine-induced hyperhomocyst(e)inaemia but completely prevented endothelial dysfunction. Our results suggest that in humans a methionine-rich diet may acutely impair endothelial function, which can be prevented by folic acid supplementation.

INTRODUCTION

Hyperhomocyst(e)inaemia is an independent risk factor for cardiovascular disease [1–3]. However, precise mechanisms responsible for the association between hyperhomocyst(e)inaemia and atherosclerosis or thrombosis are largely unknown. It has been demonstrated that diet-induced chronic hyperhomocyst(e)inaemia in primates leads to impaired vasomotor regulation in vivo and endothelial anti-thrombotic function ex vivo [4,5]. In patients with chronic hyperhomocyst(e)inaemia, endothelium-dependent vasodilatation [6,7] and endothelial anticoagulant function [8] are impaired. Thus, experimental evidence suggests that the atherogenic propensity associated with hyperhomocyst(e)inaemia results from endothelial dysfunction and injury followed by platelet activation and thrombus formation [9,10]. However, the endothelial dysfunction in chronic hyperhomocyst(e)-inaemia may be just an epiphenomenon of atherosclerosis. It has been reported that acute hyperhomocyst(e)-inaemia induced by methionine loading can impair endothelial function in humans [11]. However, Hanratty

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et al. [12] reported no changes in endothelial function by methionine loading. Thus, it is still unknown whether acute hyperhomocyst(e)inaemia induced by methionine loading also impairs endothelial function in humans.

Although precise mechanisms of homocyst(e)ine-induced endothelial dysfunction are unknown, there is growing evidence that homocyst(e)ine exerts its effects by promoting oxidative damage. It has been shown that homocyst(e)ine-induced endothelial injury in vitro is largely due to the generation of hydrogen peroxide [13,14]. Autoxidation of homocyst(e)ine produces other cytotoxic reactive oxygen species, including superoxide anion and hydroxyl radical [15–17]. Homocyst(e)ine directly decreases the bioavailability of nitric oxide (NO) by impairing its synthesis [18], and may decrease the expression of endothelial NO synthase by promoting lipid peroxidation by reactive oxygen species and degrading NO [19].

Nutritional deficiencies in the vitamin cofactors required for homocyst(e)ine metabolism may promote hyperhomocyst(e)inaemia. Markedly elevated homocyst(e)ine concentrations have been observed in patients with nutritional deficiency of folic acid [20,21]. Negative correlations between serum folic acid and plasma homocyst(e)ine [22] or cardiovascular diseases [23] have been observed. Folic acid supplementation can normalize high homocyst(e)ine concentrations [24] and may decrease cardiovascular events [25]. Furthermore, supplementation of folic acid restores in vitro endothelial function in hypercholesterolaemic subjects through reducing NO catabolism by regeneration of tetrahydrobiopterin, an essential cofactor of NO synthase [26].

Accordingly, we investigated whether acute hyperhomocyst(e)inaemia induced by oral methionine loading impairs endothelium-dependent flow-mediated vasodilatation and whether co-administration of folic acid restores methionine-induced endothelial dysfunction.

**METHODS**

**Subjects**

The study was performed in 10 healthy male volunteers, aged 26 ± 1 years. All subjects were non-smokers, non-obese (body mass index, 23 ± 1 kg/m²), non-uraemic (plasma creatinine, 1.00 ± 0.03 mg/dl), normotensive (systolic/diastolic blood pressure, 117 ± 2/72 ± 1 mmHg), non-diabetic (fasting plasma glucose, 102 ± 3 mg/dl), haemoglobin A₁c, 4.9 ± 0.2 mg/dl), normolipidaemic (total cholesterol, 164 ± 9 mg/dl; low-density lipoprotein cholesterol, 88 ± 8 mg/dl; high-density lipoprotein cholesterol, 61 ± 5 mg/dl; triacylglycerol, 78 ± 14 mg/dl) with normal plasma levels of homocyst(e)ine (7.8 ± 0.8 nmol/ml), folic acid (6.4 ± 0.3 ng/ml), vitamin B6 (16.5 ± 3.9 ng/ml) and vitamin B12 (534 ± 24 pg/ml). None of the subjects had taken any medication before the experiment.

**Study design**

The protocol was explained, and written informed consent was obtained from each subject. The study was approved by the Ethical Committee for Human Investigation at our institution. The study was performed in the morning after overnight fasting in a supine position in an air-conditioned room at a temperature of about 25 °C. We measured blood pressure, heart rate, endothelial function and plasma levels of total homocyst(e)ine at fasting and 3, 8 and 24 h after administration of either oral methionine with tap water (L-methionine, 0.1 g/kg body weight, Wako Chemicals Co., Osaka, Japan) or tap water as placebo. To examine the effects of folic acid supplementation, we repeated the above protocol to include pretreatment with folic acid (20 mg per os, Nihon Pharmaceutical Co., Tokyo, Japan) on a separate occasion. The sequence of the three experiments was alternated. Before each experiment, the subjects fasted to avoid the effects of diet.

**Measurement of endothelial function**

Flow-mediated vasodilatation of the brachial artery was measured by a previously described non-invasive technique to assess endothelial function [27]. Briefly, after a 10 min equilibration period, with use of a 10 MHz linear array transducer and SSA-380A® system (Toshiba, Tokyo, Japan), the brachial artery was longitudinally imaged at approximately 5 cm proximal to the antecubital crease, twice at baseline and then from 1 to 15 min after the release of 4.5 min of upper arm arterial occlusion at 250 mmHg of pressure with a 12.5-cm-wide cuff. Photographic images of end-diastolic frames were obtained and analysed by two independent investigators blinded to the subjects and sequences. Arterial diameter was determined by calliper measurement at the single most equivalent imaged site using side-by-side presentation. Flow-mediated (endothelium-dependent) vasodilatation was determined as the maximum percentage change of the post-occlusion arterial diameter measurement relative to the mean of the corresponding two baseline measurements. The mean of the two measurements was calculated by independent observers. The inter- and intra-observational variations of the two baseline measurements were 2.8 and 1.6% respectively. Blood flow velocity was measured by Doppler technique at baseline and immediately after the release of cuff occlusion. Arterial blood flow was determined as arterial cross-sectional area times mean Doppler velocity. The magnitude of reactive hyperaemia was calculated as the maximum flow divided by the flow during the resting scan. As an internal control we measured endothelium-independent vasodilatation induced by sublingual glyceryl trinitrate (300 mg; Myocel Spray®, Toa Eiyo Co., Tokyo, Japan) 15 min after the...
Homocyst(e)ine, folate and endothelial function measurements of flow-mediated vasodilatation. Five minutes after glyceryl trinitrate administration, a scan was performed to assess endothelium-independent vasodilatation. Flow-mediated and glyceryl trinitrate-induced vasodilations were assessed at fasting and 3, 8 and 24 h after oral methionine administration.

Chemical analysis
Total plasma concentrations of homocyst(e)ine were estimated by HPLC. Plasma folic acid and vitamin B12 were measured by a competitive protein-binding radioassay. Plasma vitamin B6 was estimated by enzymic photometry. Serum total cholesterol, triacylglycerols and creatinine were determined enzymically with commercial kits (L-type Wako cholesterol and L-type Wako TG-H respectively; Wako Chemicals Co.). High-density lipoprotein cholesterol was determined by homogeneous assay with commercial kits (Cholestest HDL, Daiichi Chemicals Co., Tokyo, Japan). Low-density lipoprotein cholesterol was calculated by the Friedewald formula. Plasma glucose was measured by the glucose dehydrogenase ultraviolet test (Merck Liquid Glu, Kanto Chemical Co., Tokyo, Japan). HbA1c was measured by latex agglutination turbidimetry using commercial kits (Rapidia Auto HbA1c, Fujirebio Inc., Tokyo, Japan).

Statistical analysis
Statistical comparisons were performed by ANOVA for repeated measures changes in endothelial function and chemical variables after methionine loading with or without folic acid. All values are expressed as means ± S.E.M., and P < 0.05 was considered to be statistically significant.

RESULTS
Blood measurements after methionine loading
Placebo did not affect plasma levels of homocyst(e)ine (pre, 6.6 ± 1.0 nmol/ml; 3 h, 7.2 ± 1.1 nmol/ml; 8 h, 7.5 ± 0.9 nmol/ml; 24 h, 6.3 ± 0.6 nmol/ml, P not significant). Methionine administration increased plasma levels of homocyst(e)ine by four times the basal level at 3 and 8 h (Figure 1; P < 0.0001, ANOVA), returning to baseline at 24 h.

Endothelial function after methionine loading
Oral administration of methionine or placebo did not affect either mean arterial pressure (placebo: pre, 85 ± 0 mmHg; 3 h, 87 ± 1 mmHg; 8 h, 86 ± 3 mmHg; 24 h, 83 ± 2 mmHg, P not significant; methionine: pre, 87 ± 1 mmHg; 3 h, 83 ± 3 mmHg; 8 h, 84 ± 2 mmHg; 24 h, 85 ± 2 mmHg, P not significant) or heart rate (placebo: pre, 67 ± 2 beats/min; 3 h, 68 ± 2 beats/min; 8 h, 68 ± 2 beats/min; 24 h, 68 ± 2 beats/min, P not significant; methionine: pre, 67 ± 2 beats/min; 3 h, 67 ± 1 beats/min; 8 h, 68 ± 1 beats/min; 24 h, 68 ± 2 beats/min, P not significant). Placebo treatment did not affect flow-mediated vasodilation (pre, 12 ± 2%; 3 h, 11 ± 12%; 8 h, 11 ± 2%; 24 h, 11 ± 2%, P not significant), glyceryl trinitrate-induced vasodilation (pre, 20 ± 6%; 3 h, 17 ± 3%; 8 h, 18 ± 4%; 24 h, 17 ± 4%, P not significant) or the magnitude of reactive hyperaemia (pre, 448 ± 26%; 3 h, 454 ± 30%; 8 h, 426 ± 26%; 24 h, 429 ± 25%, P not significant). Flow-mediated vasodilation fell by about 10% from baseline after methionine loading and returned to baseline at 24 h (Figure 1). In contrast, methionine loading did not affect glyceryl trinitrate-induced brachial artery dilation (Figure 1) and the magnitude of reactive hyperaemia (pre, 417 ± 37%; 3 h, 357 ± 33%; 8 h, 358 ± 22%; 24 h, 344 ± 38%, P not significant).

Effects of co-administration of folic acid
Co-administration of folic acid with methionine did not affect blood pressure (pre, 86 ± 1 mmHg; 3 h,
84 ± 2 mmHg; 8 h, 86 ± 2 mmHg; 24 h, 85 ± 2 mmHg, P not significant) or heart rate (pre, 65 ± 1 beats/min; 3 h, 66 ± 1 beats/min; 8 h, 67 ± 2 beats/min; 24 h, 67 ± 1 beats/min, P not significant). The plasma levels of homocyst(e)ine showed similar changes with or without co-administration of folic acid (Figure 1). The methionine-impaired flow-mediated vasodilation was abolished by co-administration of folic acid (Figure 1), but folic acid co-administration did not affect glyceryl trinitrate-induced vasodilatation (Figure 1) or the magnitude of reactive hyperaemia (pre, 409 ± 42%; 3 h, 423 ± 29%; 8 h, 460 ± 43%; 24 h, 431 ± 33%, P not significant).

DISCUSSION

The salient findings of this study are: (i) acute methionine loading decreased flow-mediated vasodilatation in parallel with the increase in plasma levels of homocyst(e)ine; and (ii) co-administration of folic acid completely prevented methionine-induced acute endothelial dysfunction.

Methionine-induced endothelial dysfunction

In this study methionine loading caused acute impairment of flow-mediated vasodilatation of the brachial artery. Our results are consistent with those of Chambers et al. [11]. They measured flow-mediated vasodilatation before and 2 and 4 h after methionine loading (0.1 g/kg, per os) at the same dose that we used in normal subjects and found a close association between the increase in plasma homocyst(e)ine and the impairment of vasodilatation. The increases in plasma homocyst(e)ine levels were similar in both studies. In addition, we clearly demonstrated that the impaired flow-mediated vasodilatation was restored 24 h later when the plasma homocyst(e)ine level returned to the baseline level. Methionine loading did not affect glyceryl trinitrate-induced vasodilatation. Flow-mediated vasodilatation during reactive hyperaemia is considered to be endothelium-derived and NO-dependent [27]. Thus our results suggest that acute hyperhomocyst(e)inaemia may have impaired the bioactivity of NO. Indeed, we demonstrated that pretreatment with the NO synthase inhibitor $N^{\omega}$-monomethyl-l-arginine, infused into the brachial artery, completely abolished the flow-mediated vasodilatation in healthy volunteers [28]. Taken together, acute hyperhomocyst(e)inaemia induced by methionine loading impairs endothelium-derived NO-dependent vasodilatation, which is reversible 24 h later.

Because methionine loading increases the plasma level of methionine as well as homocyst(e)ine, the consequent endothelial dysfunction may be due to increased plasma methionine rather than homocyst(e)ine. However, this possibility is less likely because methionine itself is not as toxic as homocyst(e)ine for cultured endothelial cells [29]. In addition, the time course of plasma homocyst(e)ine and methionine levels after methionine loading is different. The concentrations of homocyst(e)ine reach their peak level at 6–8 h after methionine loading, the point at which endothelium-dependent vasodilatation is maximally impaired, whereas it has been reported that plasma methionine reaches its peak level at 2–3 h after methionine loading [30], indicating that endothelial function is more closely associated with the plasma levels of homocyst(e)ine than those of methionine.

Effects of folic acid

It has been reported that chronic administration of folic acid decreases plasma levels of homocyst(e)ine [24,25]. We anticipated that acute supplementation of folic acid might attenuate the increase in plasma homocyst(e)ine induced by methionine loading. Contrary to our expectation, the peak level of homocyst(e)ine was unchanged when folic acid was co-administered. Nonetheless, endothelium-dependent vasodilatation was restored by folic acid supplementation. Apart from its homocyst(e)ine-lowering effect, folic acid has been suggested to stimulate endogenous tetrahydrobiopterin, an essential cofactor of NO synthase, regenerated from inactive dihydrobiopterin [31]. Verhaar et al. [26] demonstrated that administration of 5-methyltetrahydrofolate, an active form of folic acid, restored impaired endothelial function in patients with hypercholesterolaemia. Furthermore, experiments in vitro have shown that 5-methyltetrahydrofolate reduces superoxide generation from NO synthase and xanthine oxidase [26]. Collectively, it is possible that acute hyperhomocyst(e)inaemia may impair endothelial function by oxidative stress and that folic acid administration may prevent endothelial dysfunction via amelioration of oxidative stress.

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