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Folic acid treatment reduces elevated plasma levels of asymmetric dimethylarginine in hyperhomocysteinaemic subjects

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Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, has been suggested to be a novel risk factor for endothelial dysfunction. It has previously been reported that hyperhomocysteinaemia may be associated with impaired endothelium-dependent vasodilation and reduced plasma level of NO-derived endproducts (NOx). In the present study, plasma levels of arginine and ADMA were measured in twenty-one healthy control subjects, and in twenty-one hyperhomocysteinaemic subjects before and after 6 weeks and 12 months of folic acid supplementation, and compared with previously measured plasma NOx values in the hyperhomocysteinaemic subjects. Compared with control subjects, hyperhomocysteinaemic subjects had higher plasma levels of arginine and ADMA. More importantly, folic acid therapy significantly reduced plasma levels of arginine and ADMA. Furthermore, plasma levels of arginine and ADMA were positively correlated with plasma homocysteine levels and negatively correlated with plasma folate, as well as negatively correlated with plasma NOx. Our results suggest that ADMA may be a mediator of the atherogenic effects of homocysteine.

Homocysteine: Folic acid: Asymmetric dimethylarginine: Nitric-oxide-derived endproducts

Asymmetric dimethylarginine (ADMA) has been suggested to be a novel risk factor for endothelial dysfunction (Böger *et al.* 1998). ADMA is an endogenous competitive inhibitor of NO synthase (NOS), the enzyme catalysing the formation of NO from arginine (Böger *et al.* 1998). Plasma levels of ADMA have been shown to be elevated in various cardiovascular disorders, such as atherosclerosis (Miyazaki *et al.* 1991), hypertension (Matsuoka *et al.* 1997) and congestive heart failure (Feng *et al.* 1998). In addition, raised plasma levels of ADMA have been reported in monkeys with hyperhomocysteinaemia and/or hypercholesterolaemia (Böger *et al.* 2000*a*), in healthy subjects following an oral methionine load (Böger *et al.* 2001) and recently also in elderly patients with stroke (positively correlated with plasma homocysteine levels in these patients (Yoo & Lee, 2001).

Hyperhomocysteinaemia is an independent risk factor for cardiovascular disease (Harker *et al.* 1974; Ueland *et al.* 1992; Boushey *et al.* 1995). While the mechanisms by which homocysteine may cause vascular lesions have not yet been fully characterised (Kokame *et al.* 1996; Upchurch *et al.* 1997; Wang *et al.* 1997), several studies suggest that homocysteine may promote endothelial dysfunction (Lentz *et al.* 1996; Tawakol *et al.* 1997; Woo *et al.* 1997; Bellamy *et al.* 1998; Chambers *et al.* 1998). Elevated concentrations of homocysteine can be reduced effectively by folic acid supplementation (Brattstrom *et al.* 1988), and such therapy has also been shown to improve endothelium-dependent vasodilation (Bellamy *et al.* 1999; Usui *et al.* 1999; Woo *et al.* 1999; Wilmink *et al.* 2000), possibly involving NO-related mechanisms (Holven *et al.* 2001).

Abbreviations: ADMA, asymmetric dimethylarginine; NOS, nitric-oxide synthase; NOx, nitric-oxide-derived endproduct.

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To elucidate further the possible interactions between homocysteine, NO and its endogenous NOS inhibitor ADMA, in the present study we examined: (1) plasma level of the NO-precursor arginine and ADMA in hyperhomocysteinaemic subjects and healthy controls; (2) the effect of folic acid supplementation on arginine and ADMA levels in subjects with hyperhomocysteinaemia.

Methods

Subjects

Twenty-one adults, <75 years of age, with hyperhomocysteinaemia (fasting plasma total homocysteine concentration >15 μmol/l) were recruited at the Lipid Clinic, Rikshospitalet, Oslo, Norway. Three of the participants had hypertension, two had established coronary artery disease, ten were on statin treatment and eleven were current smokers. Control subjects (*n* 21) were age-and sex-matched healthy non-smoking volunteers with no history of hypertension, diabetes, coronary artery disease or other acute or chronic illness. 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 subjects. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Intervention with folic acid

The study was an uncontrolled open supplementation trial, and the study protocol has previously been reported (Holven *et al.* 2001). Briefly, patients with hyperhomocysteinaemia received, after baseline evaluation, 5 mg folic acid/d during the first week, then 1 mg folic acid/d for the following 37 weeks, and finally 400 μg folic acid/d for the last 14 weeks. All but one patient was included during a 2-week period. All patients had previously been instructed by a nutritionist to follow the National Cholesterol Education Program Step I Diet (Department of Health and Human Services, 1993) and this diet was followed throughout the study. All patients were followed for the first period (6 weeks), and thirteen of the patients completed the study (12 months). At baseline, and after 6 weeks and 12 months of folic acid treatment, venous blood samples were collected after an overnight fast. Plasma and serum samples were stored at -80°C.

Concentration of arginine and asymmetric dimethylarginine

Plasma samples were cleaned and deproteinised by refrigerated (4°C) ultracentrifugation through a 3×10^4 Da cut-off filter, and stored at -80°C until analysis. Amino acids were measured by HPLC (Perkin Elmer Wallac Oy, Turku, Finland). In brief, the amino acids were automatically pre-column derivatised by an autosampler (Perkin Elmer ISS 200) with 40 mM-*o*-phthalaldehyde (Fluka Chemie AG/Sigma Aldrich, St Louis, MO, USA) and 3-mercapto propionic acid (Sigma, St Louis, MO, USA) at pH 10·5. The amino acids were separated on a reversed phase Hypersil ODS column (3·1 × 200 mm; Chrompak International B.W., Varian Inc, Palo Alto, CA, USA) at 42°C by gradient elution (Perkin Elmer Binary LC pump 250). Mobile phases were: A 0·08 M-sodium acetate (Sigma) buffer, pH 6·0, B acetonitrile (Rathburn Chemicals Ltd, Peebleshire, UK)–0·4 M-sodium acetate buffer–methanol (Rathburn Chemicals Ltd) (14:4:1, by vol.). Fluorescence peaks (Perkin Elmer LS 50B; excitation wavelength 340 nm, emission wavelength 460 nm) were integrated by Perkin Elmer 1020X software on a PE Nelson computer (Perkin Elmer, Shelton, CT, USA).

Concentration of nitric-oxide-derived endproducts

Plasma concentration of the NO-derived endproducts (NOx), nitrite and nitrate, were measured by enzyme immunoassays from R&D Systems (Minneapolis, MN, USA), as previously described (Holven *et al.* 2001). The inter and intra-assay CV were <7 %. To minimise interference with plasma proteins, the samples were ultrafiltered through a 10 kDa cut-off filter (Microcon YM-10; Millipore Corp., Bedford, MA, USA) prior to the analysis of nitrite and nitrate.

Concentration of total homocysteine

Plasma concentration of total homocysteine was determined by HPLC (Ubbink *et al.* 1991).

Routine laboratory assays

Vitamin B_{12} and folate were measured using the fluoroimmunoassay autoDELFIA™ B12 and autoDELFIA™ Folate respectively (Perkin Elmer; inter-and intra-assay CV <9 % for both assays). Triacylglycerol, total cholesterol and HDL- and LDL-cholesterol were measured using enzymatic colorimetric tests (Roche Diagnostics, Indianapolis, IN, USA; inter-and intra-assay CV <3 % for all assays); creatinine was measured using a kinetic colorimetric assay (Roche Diagnostics; inter-and intra-assay CV $<2{\cdot}5$ %).

Statistical methods

Data are given as mean values and standard deviations. At baseline, patients and controls were compared by the Student's *t* test. When the data were not normally distributed, they were subjected to logarithmic transformation before the parametric analysis. Univariate ANOVA compared pre- and post-treatment values in the patient group. Bonferroni's test was used for *post-hoc* analysis when the ANOVA was positive. Associations between variables were tested by Pearson correlation coefficients. The level of statistical significance was set at $P<0{\cdot}05$.

Results

Characteristics of the hyperhomocysteinaemic subjects

The baseline characteristics of the hyperhomocysteinaemic subjects and the healthy controls are given in Table 1. Compared with healthy control subjects, patients with hyperhomocysteinaemia had significantly raised plasma

Table 1. Plasma concentrations of homocysteine, vitamins, lipids, creatinine and nitric-oxide-derived endproducts (NOx) in control subjects and in hyperhomocysteinaemic subjects before, and after 6 weeks and 12 months, folic acid treatment‖

(Mean values and standard deviations)

	Control subjects		Hyperhomocysteinaemic subjects					
	Baseline (*n* 21)		Baseline (*n* 21)		6 weeks (*n* 21)		12 months (*n* 13)	
Characteristic	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Female (*n*) male (*n*)	7/14		7/14					
Age (years)	46·7	10·3	45·8	16·3				
Homocysteine (μmol/l)	11·0	1·9	21·0***	10·8	9·8†††	1·7	10·8†††	1·7
Folate (nmol/l)	12·8	7·6	9·5	5·5	56·1†††	28·7	35·9†††	13·2
Vitamin B_{12} (pmol/l)	279	90	206	57	208	64	279§	32
Cholesterol (mmol/l)	5·0	0·6	5·7	1·6	5·6	1·3	5·6	1·3
HDL-cholesterol (mmol/l)	1·3	0·4	1·3	0·3	1·3	0·3	1·1	0·2
LDL-cholesterol (mmol/l)	3·3	0·5	3·8	1·5	3·7	1·3	4·1	1·2
Triacylglycerol (mmol/l)	1·1	0·5	1·3	0·6	1·3	0·7	1·1	0·5
NOx (μmol/l)¶	29	10	14***	9	18	8	25††	9
Creatinine (μmol/l)	85	11	82	10	86	10	84	10

Mean values were significantly different from those of the control subjects: ***$P<0·001$.
Mean values were significantly different from those of hyperhomocysteinaemic subjects at baseline: ††$P<0·003$, †††$P<0·001$.
Mean values were significantly different from those of hyperhomocysteinaemic subjects at baseline and after 6 weeks treatment: §$P<0·05$.
‖ For details of subjects and procedures, see p. 360.
¶ Control subjects *n* 13, hyperhomocysteinaemic subjects *n* 19 at baseline and after 6 weeks treatment.

homocysteine ($P<0·001$, Table 1). More importantly, plasma ADMA level was significantly increased ($P<0·001$, Fig. 1), whereas plasma NOx was significantly reduced ($P<0·001$, Table 1). Plasma arginine levels were also increased in patients with hyperhomocysteinaemia, although the difference did not reach statistical significance ($P=0·058$; Fig. 2). Among patients with hyperhomocysteinaemia, there was no significant difference in plasma ADMA, arginine or NOx levels between statin-users and non-statin-users, smokers and non-smokers, or females and males (results not shown).

Effect of folic acid supplementation

Compared with baseline levels, folic acid supplementation of the hyperhomocysteinaemic subjects for 6 weeks and 12 months significantly reduced the plasma level of homocysteine ($P<0·001$ *v.* baseline, Table 1), and increased serum concentration of folate ($P\leq0·001$ *v.* baseline, Table 1). Notably, these changes in plasma homocysteine and folate in this group of hyperhomocysteinaemic patients were accompanied by a marked decrease in both ADMA and arginine levels (Figs. 1 and 2) as well as a marked

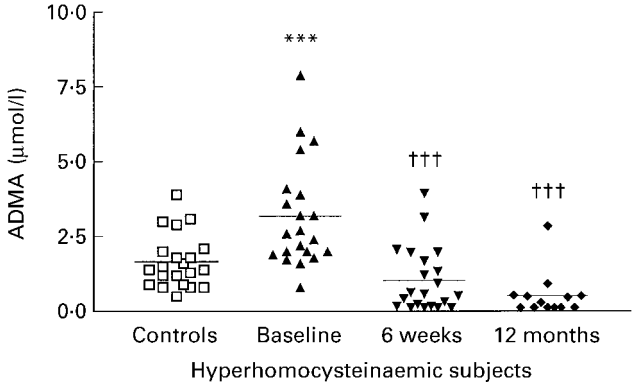

Fig. 1. Plasma concentrations of asymmetric dimethylarginine (ADMA) in healthy controls at baseline (□, *n* 21), and in hyperhomocysteinaemic subjects at baseline (▲, *n* 21), and after 6 weeks (▼, *n* 21) and 12 months (◆, *n* 13), folic acid treatment. For details of subjects and procedures, see p. 360. Mean values are shown by horizontal bars. Mean value was significantly different from that of the control subjects: ***$P<0·001$. Mean values were significantly different from those of hyperhomocysteinaemic subjects at baseline: †††$P<0·001$.

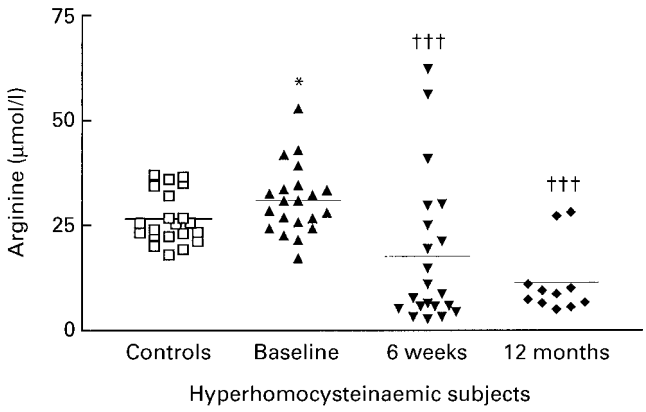

Fig. 2. Plasma concentrations of arginine in healthy controls at baseline (□, *n* 21), and in hyperhomocysteinaemic subjects at baseline (▲, *n* 21), and after 6 weeks (▼, *n* 21) and 12 months (◆, *n* 13), folic acid treatment. For details of subjects and procedures, see p. 360. Mean values are shown by horizontal bars. Mean value was significantly different from that of the control subjects: *$P<0·058$. Mean values were significantly different from those of hyperhomocysteinaemic subjects at baseline: †††$P<0·001$.

increase in plasma NOx (Table 1). Furthermore, in the hyperhomocysteinaemic group, plasma levels of arginine and ADMA were positively correlated with plasma level of homocysteine (r 0·39, $P<0·01$ and r 0·43, $P<0·001$ respectively), and negatively correlated with plasma folate (r −0·42, $P<0·001$ and r −0·46, $P<0·001$ respectively) and plasma NOx (r −0·33, $P<0·05$ and r −0·35, $P<0·02$ respectively). Plasma arginine and ADMA were also positively correlated (r 0·755, $P<0·001$). The correlation analyses were based upon measurements at all three timepoints in the study.

Discussion

ADMA has been suggested to be a novel risk factor for endothelial dysfunction (Böger *et al.* 1998). In the present study, we have shown that hyperhomocysteinaemic individuals are characterised by raised plasma levels of ADMA, positively correlated with homocysteine and negatively correlated with folate levels. Even more importantly, the reduction in homocysteine levels during folic acid supplementation was accompanied by a significant decrease in plasma levels of this endogenous NOS inhibitor. Our results suggest that ADMA may be a mediator of the atherogenic effects of homocysteine.

Endothelium-derived NO is a potent endogenous vasodilator (Furchgott & Zawadzki, 1980), and we have previously shown that these hyperhomocysteinaemic individuals have decreased plasma levels of NOx (Holven *et al.* 2001), suggesting impaired NO activity in these patients. Several factors may contribute to these disturbances, but the present study suggests that the decreased NOx level is not caused by decreased availability of the NO precursor arginine. In fact, hyperhomocysteinaemic patients had increased plasma levels of this amino acid. However, these patients also had raised ADMA levels negatively correlated with plasma levels of NOx, suggesting that, rather than decreased precursor availability, the reduction in NOx may at least in part be due to an increased plasma level of the endogenous NOS inhibitor ADMA. Elevated ADMA level has been shown in patients with atherosclerosis (Miyazaki *et al.* 1991), hypertension (Matsuoka *et al.* 1997) and congestive heart failure (Feng *et al.* 1998), as well as in monkeys with hyperhomocysteinaemia and/or hypercholesterolaemia (Böger *et al.* 2000*a*). It has also been reported in healthy subjects following an oral methionine load (Böger *et al.* 2001), and in elderly patients with stroke (positively correlated with plasma homocysteine in these patients (Yoo & Lee, 2001)). Although the molecular mechanisms leading to raised ADMA levels in these disorders have not been fully elucidated, Stuhlinger *et al.* (2001) recently presented evidence suggesting that homocysteine may impair the NOS pathway directly by inhibiting dimethylarginine dimethylaminohydrolase activity, thereby causing ADMA accumulation, resulting in the subsequent reduction in NO synthesis. Whatever the mechanisms, evidence is accumulating that ADMA is one possible cause of endothelial dysfunction in several disorders, and it is tempting to speculate that an ADMA-mediated inhibition of NO synthesis may contribute to the impaired endothelium-dependent vasodilation previously shown in hyperhomocysteinaemic patients (Tawakol *et al.* 1997; Woo *et al.* 1997; Bellamy *et al.* 1998, 1999; Chambers *et al.* 1998; Holven *et al.* 2001). However, our present findings do not prove any causal relationship between ADMA and endothelial dysfunction in patients with hyperhomocysteinaemia and the exact role of ADMA in these patients will have to be further elucidated.

A major and novel finding in the present study was a marked reduction in ADMA levels during folic acid supplementation in hyperhomocysteinaemic individuals. We have previously shown that folic acid treatment in this group of hyperhomocysteinaemic subjects leads to a marked increase in plasma NOx levels as well as an improved endothelium-dependent vasodilation (Holven *et al.* 2001). Moreover, folic acid therapy also induced a significant reduction in arginine levels. Although the reason for this decrease has not been clarified, it may reflect increased consumption of this NO precursor after removal of an ADMA-induced inhibition of NOS. Notably, Böger *et al.* (2000*a*) have recently reported that while vitamin B supplementation decreased homocysteine level in hyperhomocysteinaemic monkeys, these vitamins had no effect on ADMA level or endothelial dysfunction. Although that experiment may not be comparable with the present study, a direct effect of folic acid on NO and ADMA metabolism should be further investigated in human subjects.

In the present study, we have shown that patients with hyperhomocysteinaemia are characterised by high levels of the endogenous NOS inhibitor ADMA. Moreover, folic acid supplementation significantly decreased plasma levels of ADMA, suggesting the involvement of ADMA-mediated mechanisms in the decreased NO activity in these patients. While the inhibitory effects on NOS is best studied, ADMA seems to have additional effects on endothelial cells with potential atherogenic properties such as enhancement of monocyte adhesiveness, augmented chemokine synthesis and increased oxidative stress with secondary activation of the transcriptional factor nuclear factor-κB (Böger *et al.* 2000*b*). Thus, ADMA has been proposed to be an endogenous proatherogenic molecule, and our present findings suggest that ADMA may represent a 'novel' pathway for the homocysteine-mediated atherogenic effects, possibly involving NO-related mechanisms.

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