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ADMA and oxidative stress are responsible for endothelial dysfunction in hyperhomocyst(e)inemia: effects of L-arginine and B vitamins☆

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Abstract

Objectives: Hyperhomocyst(e)inemia is a risk factor for atherosclerotic vascular disease, and it is associated with endothelial dysfunction. Mechanisms responsible for endothelial dysfunction in hyperhomocyst(e)inemia may involve impaired bioavailability of NO, possibly secondary to accumulation of the endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA) and increased oxidative stress. We investigated whether oral treatment with B vitamins or L-arginine normalizes endothelium-dependent, flow-dependent vasodilation (FDD) in patients with peripheral arterial occlusive disease (PAOD) and hyperhomocyst(e)inemia. **Methods:** 27 patients with PAOD and hyperhomocyst(e)inemia were assigned to oral treatment with combined B vitamins (folate, 10 mg; vitamin B-12, 200 μg; vitamin B-6, 20 mg/day), L-arginine (24 g/day) or placebo, for 8 weeks in a double-blind fashion. FDD was determined by high-resolution ultrasound in the radial artery. **Results:** Vitamin B supplementation significantly lowered plasma homocyst(e)ine concentration from 15.8±1.8 to 8.7±1.1 μmol/l ($P<0.01$). However, B vitamins had no significant effect on FDD (baseline, 7.8±0.7%, B vitamins, 8.3±0.9%, placebo 8.9±0.7%; P=n.s.). In contrast, L-arginine treatment did not affect homocyst(e)ine levels, but significantly improved FDD (10.2±0.2%), probably by antagonizing the impact of elevated ADMA concentration (3.8±0.3 μmol/l) and reducing the oxidative stress by lowering urinary 8-iso-prostaglandin $F_{2\alpha}$ (baseline, 76.3±7.1 vs. 62.7±8.3 pmol/mmol creatinine after 8 weeks). **Conclusions:** Oral supplementation with combined B vitamins during 8 weeks does not improve endothelium-dependent vasodilation in PAOD patients with hyperhomocyst(e)inemia, whereas L-arginine significantly improved endothelial function in these patients. Thus, accumulation of ADMA and increased oxidative stress may underlie endothelial dysfunction under hyperhomocyst(e)inemic conditions. These findings may have importance for evaluation of homocyst(e)ine-lowering therapy.

Keywords: Atherosclerosis; Endothelial function; Nitric oxide

1. Introduction

Elevated homocyst(e)ine concentrations have been identified as an independent cardiovascular risk factor [1–3]. Hyperhomocyst(e)inemia is common in patients with peripheral arterial occlusive disease (PAOD), and it is associated with progression of the disease [4,5]. Hyperhomocyst(e)inemia may be due to inherited enzyme defects, renal insufficiency, or inherited or acquired defects in homocysteine metabolism [6]. B vitamins are essential cofactors for the transsulfuration of homocysteine to cystathionine by cystathionine β-synthase (vitamin B-6), and the remethylation of homocysteine to methionine by methionine synthase (folic acid, vitamin B-12). Several studies have demonstrated that dietary supplementation

☆The term 'hyperhomocyst(e)inemia' is used in this paper to indicate that plasma homocysteine assays measure the total concentration of thiol, disulfide, and mixed disulfide adducts of homocysteine.

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with combined B vitamins is an efficient means to decrease plasma homocyst(e)ine [7]. However, the results of prospective studies investigating potential beneficial effects of B vitamin supplementation on vascular function in patients with PAOD and hyperhomocyst(e)inemia are lacking [3].

The mechanism(s) by which homocysteine contributes to vascular disease remain poorly understood. Homocysteine alters the normal antithrombotic properties of the vascular wall by modulating the activity of the coagulant and fibrinolytic systems, and promotes vascular smooth muscle cell proliferation [8]. Furthermore, there is evidence that hyperhomocyst(e)inemia is associated with endothelial dysfunction in animals and humans [9–11]. Mechanisms may involve reduced elaboration of NO by the endothelium or oxidative inactivation of NO. Homocysteine increases the oxidative degradation of NO through the formation of disulfides and the generation of hydrogen peroxide and superoxide anion [12,13]. In a recent study we found that cynomolgus monkeys fed a methionine-rich diet showed elevated plasma levels of asymmetric dimethylarginine (ADMA) [14], an endogenous NO synthase inhibitor [15]. Lowering homocysteine levels with B vitamins in these animals did not improve endothelial function, which was closely correlated to ADMA plasma concentration [14]. Furthermore, a methyl group which is cleaved during synthesis of homocysteine from methionine is utilized during ADMA synthesis in cultured human endothelial cells, thereby linking homocysteine and ADMA metabolic pathways [16].

In the present study we investigated whether supplementation with folic acid, vitamin B-12, and vitamin B-6 reduces elevated homocyst(e)ine plasma levels and improves endothelium-dependent, NO-mediated vasodilation and vascular functional status in PAOD patients. We also studied whether ADMA concentration and urinary excretion of 8-iso-prostaglandin-$F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$)—a non-invasive marker for oxidative stress in vivo—are elevated, and whether oral L-arginine supplementation improves endothelial function in these patients. Improved endothelial function is associated with reduced progression of cardiovascular disease [17], and may therefore indicate an overall beneficial effect of these treatments on atherosclerosis in patients with cardiovascular disease and high homocyst(e)ine plasma levels.

2. Methods

2.1. Patients and study protocol

Twenty-seven patients with PAOD and stable intermittent claudication who had elevated homocyst(e)ine plasma concentration gave their written informed consent to participate in the study. All patients had been on a stable medication before the beginning of the study, and doses remained unchanged throughout the study. With the exception of acetylsalicylic acid, all medication was stopped 24 h before measurements. None of the participants were taking additional vitamin supplements as assessed at the baseline. They were all advised not to start vitamin supplementation while the study was ongoing. Alcohol and caffeine were prohibited within 12 h of the study. At baseline, a venous blood sample was taken under fasting conditions for the measurement of plasma total homocyst(e)ine, B vitamin, L-arginine, ADMA and symmetric dimethylarginine (SDMA) levels. A urine sample was collected to measure urinary excretion of 8-iso-$PGF_{2\alpha}$. Endothelium-dependent and -independent vasodilation was determined in the radial artery of the non-dominant arm using a highly sensitive and specific ultrasonic method as described below. Baseline data on flow-mediated vasodilation were also obtained in a group of 12 age-matched healthy controls (eight men; age, 73.4±0.7 years). Systolic ankle pressures were measured by Doppler in the dorsal foot artery and the posterior tibial artery. Systolic and diastolic arterial blood pressure was measured at the ipsilateral arm with the standard sphygmomanometric method. Patients were randomized to receive either a mixture of B vitamins folic acid (10 mg/day), vitamin B-6 (pyridoxal 5′-phosphate, 20 mg/day), vitamin B-12 (cyanocobalamin, 200 μg/day), L-arginine (24 g/day), or placebo, in three daily doses, for 8 weeks in a double-blind fashion. Randomization and labelling of the visually identical medication vials was performed by the local pharmacy, and the randomization code was strictly concealed from the investigators until the end of the study. After 4 and 8 weeks of treatment additional sets of blood and urine samples were collected, and blood pressure measurements, and endothelial function study were repeated. One patient in each group withdrew consent to participate after the baseline visit. The investigation conforms with the principles outlined in the Declaration of Helsinki. The study protocol had previously been approved by the Institutional Review Board for Studies in Humans at Hannover Medical School. Characteristics of the patients are shown in Table 1.

2.2. Endothelium-dependent and -independent vasodilation

Radial artery diameters were measured after an overnight fasting period by a high-resolution A-mode ultrasonic echo-tracking device (ASULAB) by a 10 MHz transducer that allows measurements of arterial diameter with a precision of ±2.5 μm [18]. Forearm blood flow was measured continuously by an 8-MHz Doppler probe (Vasoscope III) 5 cm proximal to the 10-MHz probe. Arterial blood flow (ml/min) at the mid-forearm level was calculated as the product of blood flow velocity and cross-sectional area. Wrist arterial occlusion was performed by inflating an occlusion cuff to 40 mmHg above systolic blood pressure for 8 min. After release of arterial occlu-

Table 1
Patient characteristics

	B Vitamins	L-Arginine	Placebo
N	9	9	9
Sex (m/f)	7/2	8/1	7/2
Age (years)	69.3 (4.0)	64.0 (4.1)	68.8 (2.8)
Height (cm)	170.2 (3.0)	171.6 (2.0)	171.3 (3.5)
Weight (kg)	77.6 (5.9)	76.9 (3.6)	79.3 (3.3)
Occlusion type (*n*)			
Iliaco-femoral	4	6	4
Multisegmental	5	3	5
Cardiovascular risk factors (*n*)			
Hypercholesterolemia	6	5	4
Hypertension	7	6	6
History of smoking	8	8	8
Diabetes mellitus	1	4	1

Data are mean±S.E.M. or number of patients (*N*) as indicated. There were no significant differences between the groups in any of the parameters.

sion, arterial diameter was determined until diameter returned to baseline. Maximal flow-mediated vasodilation was used for further data analysis; it occurred at 62±4 s. After measurement of flow-dependent vasodilation, 30 min of rest was allowed. Thereafter, endothelium-independent vasodilation was assessed as the vasodilator response to sublingual nitroglycerine (0.4 mg). Maximal vasodilation to nitroglycerine occurred at 4.2±0.7 min; this value was used in statistical data analysis. Reproducibility and variability of the method were very good as previously determined in our laboratory [19].

2.3. Biochemical analyses

Blood samples were obtained after an overnight fasting period and were drawn into pre-chilled vacutainers containing EDTA, and immediately centrifuged. Plasma was stored at −20 °C in appropriate aliquots until analyses.

Fasting plasma homocyst(e)ine concentrations were measured by high-performance liquid chromatography based upon the modification described by Ubbink et al. [20] of the method of Araki and Sako [21]. Briefly, plasma or standards were incubated with tri-*n*-butylphosphine in dimethylformamide (10% v/v) for 30 min (4 °C) to accomplish reduction of homocyst(e)ine and its release from protein binding. Subsequently, protein was precipitated with trichloroacetic acid. After centrifugation, the clear supernatant was derivatized with ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulphonate solution. The mixture was incubated for 1 h at 60 °C to accomplish complete derivatization of homocyst(e)ine, and subsequently used for HPLC analysis.

Plasma concentrations of vitamin B-12 and folic acid were measured by a commercially available fluorescence polarisation assay (Abbott IMX®, Abbott Diagnostics, Germany). Plasma vitamin B-6 levels were measured radioenzymatically by the method of Camp et al. [22].

Plasma concentrations of L-arginine, ADMA, and SDMA were determined by HPLC using pre-column derivatization with *o*-phthaldialdehyde (OPA) as described previously [23].

Urinary 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) excretion was determined from a morning urine sample by gas chromatography–tandem mass spectrometry [24].

Plasma total cholesterol and triglyceride concentrations were determined spectrophotometrically using standard laboratory methods.

2.4. Calculations and statistical analyses

Based on previous studies [10,11], we calculated that a sample size of at least eight patients per group was needed to detect a 2.5% increase in endothelium-dependent vasodilation with an estimated S.D. of 1.5% [11] with 90% power and a significance level of 0.05. Nine patients were included in each treatment group; after drop-outs, complete data sets were available for eight patients per group which were analyzed on an on-treatment basis. Data are given as mean±S.E.M. Statistical significance was tested using analysis of variance (ANOVA) for repeated measurements using the Bonferroni–Dunn correction. Statistical significance was accepted for $P<0.05$.

3. Results

3.1. Endothelial function

Baseline diameter of the radial artery was 3.039±0.084 mm in the B vitamin group, 3.145±0.088 mm in the L-arginine group, and 3.214±0.105 mm in the placebo group (P=n.s. between groups). After 8 weeks, there was no significant difference in baseline arterial diameter in either of the groups (B vitamins, 3.084±0.084 mm; L-arginine, 3.089±0.096 mm; placebo, 3.194±0.106 mm; P=n.s.). Intraday variability of the measurements was 0.4±0.2% at the baseline visit and 0.4±0.1% at 8 weeks. Reproducibility of diameter measurements between baseline and week 8 was 1.19±0.46%.

Mean flow-induced, NO-mediated vasodilation of the radial artery was 7.6±0.5% at baseline with no significant difference between the groups, as compared to 12.3±0.9% in age-matched healthy controls ($P<0.05$; Fig. 1). It was not significantly changed by B vitamins (7.8±1.0% vs. 8.3±0.9%) or by placebo (7.7±1.1% vs. 8.9±0.7%) in hyperhomocyst(e)inemic patients after 8 weeks of treatment. By contrast, supplementation with L-arginine significantly enhanced endothelium-dependent vasodilation (7.2±0.9% vs. 10.2±0.9%; $P<0.05$ vs. baseline and vs. placebo; Fig. 1). Endothelium-independent vasodilation induced by nitroglycerine remained unchanged during the

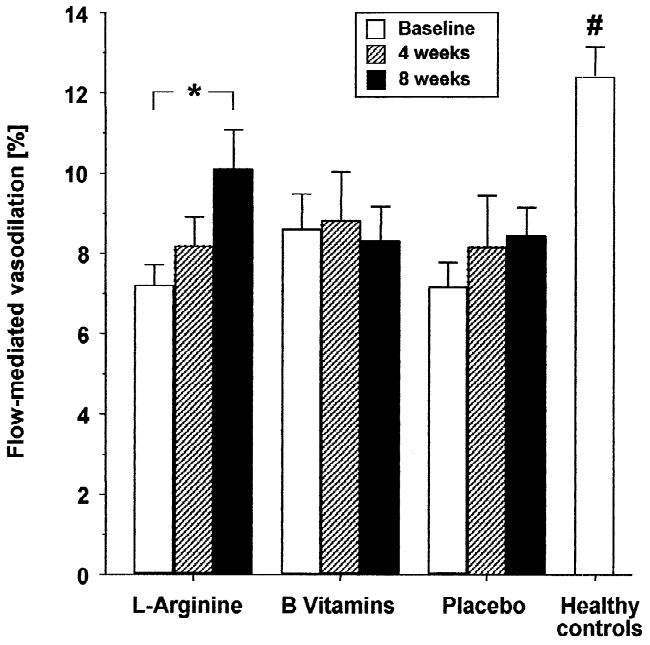

Fig. 1. Flow-dependent, NO-mediated vasodilation in the radial artery in patients with PAOD and hyperhomocyst(e)inemia, as compared to a group of healthy, age-matched controls ($N=12$; 71.8±0.5 years). Data are mean±S.E.M. of $N=8$ patients per treatment group. *$P<0.05$ vs. baseline. #$P<0.05$ vs. hyperhomocyst(e)inemic patients at baseline.

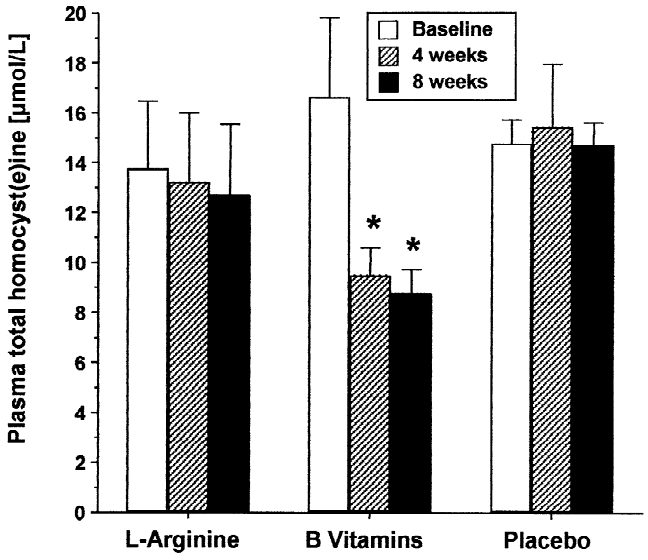

Fig. 2. Plasma total homocyst(e)ine concentration in patients with PAOD and hyperhomocyst(e)inemia. Data are mean±S.E.M. of $N=8$ patients per treatment group. *$P<0.05$ vs. baseline.

study period in each of the three treatment groups (Table 2).

3.2. Biochemical assays

Plasma total homocyst(e)ine levels at baseline were 15.0±1.4 μmol/l (mean±S.E.M.) with no significant difference between the groups. After treatment with B vitamins for 8 weeks, plasma homocyst(e)ine concentration was significantly decreased to 8.7±1.1 μmol/l ($P<0.01$; Fig. 2). Neither L-arginine nor placebo had a significant effect on homocyst(e)ine levels (Fig. 2).

Plasma concentrations of vitamins B-6, B-12, and folic acid were significantly elevated by B vitamin supplementation (each $P<0.001$ vs. baseline; $P<0.001$ vs. placebo), but unaffected by L-arginine or placebo (Table 3).

Plasma concentrations of ADMA were significantly elevated as compared to levels reported for healthy subjects of the same age (3.8±0.3 μmol/l vs. 1.0±0.1 μmol/l) [25]. None of the treatments exerted a significant effect on ADMA or SDMA concentrations (Table 3). The mean L-arginine levels of the three groups were 43.1±3.0 μmol/l at baseline. They increased to 70.9±12.6 μmol/l during L-arginine supplementation ($P<0.05$), and were unchanged in the other two groups (Table 3). L-Arginine/ADMA ratio was significantly increased by L-arginine supplementation, but unaffected in the other groups (Fig. 3).

Urinary excretion of 8-iso-$PGF_{2\alpha}$ was 73.8±5.5 pmol/mmol creatinine at baseline. It was significantly reduced during L-arginine treatment (62.7±8.3 pmol/mmol

Table 2
Changes in functional parameters during treatments

	B Vitamins		L-Arginine		Placebo	
	Baseline	8 weeks	Baseline	8 weeks	Baseline	8 weeks
NTG-induced vasodilation (%)	17 (2)	16 (2)	19 (2)	15 (2)	15 (2)	14 (2)
Systolic blood pressure (mmHg)	165 (14)	155 (12)	162 (7)	158 (5)	164 (6)	164 (5)
Diastolic blood pressure (mmHg)	93 (6)	91 (5)	86 (3)	84 (3)	83 (2)	82 (2)
Ankle blood pressure (mmHg)	124 (16)	105 (12)	140 (8)	132 (15)	133 (12)	140 (12)
ABPI	0.7 (0.1)	0.7 (0.1)	0.8 (0.1)	0.8 (0.1)	0.7 (0.1)	0.8 (0.1)

Data are mean±S.E.M. NTG, nitroglycerine; ABPI, ankle-brachial pressure index. There were no significant differences between the groups in any of the parameters.

Table 3
Changes in biochemical parameters during treatments

	B Vitamins		L-Arginine		Placebo	
	Baseline	8 weeks	Baseline	8 weeks	Baseline	8 weeks
Folic acid (nmol/l)	14.6 (1.6)	173.1* (44.4)	10.3 (0.6)	11.6 (1.0)	12.7 (1.3)	13.4 (1.5)
Vitamin B-6 (nmol/l)	17.9 (2.5)	140.2* (19.1)	32.7 (9.3)	27.5 (5.6)	26.7 (7.3)	23.6 (7.0)
Vitamin B-12 (pmol/l)	230 (32)	800* (156)	240 (47)	215 (39)	210 (34)	183 (19)
ADMA (μmol/l)	4.1 (0.5)	3.9 (0.4)	3.5 (0.3)	3.1 (0.4)	3.9 (0.7)	3.6 (0.6)
SDMA (μmol/l)	2.3 (0.3)	1.9 (0.4)	2.2 (0.4)	2.2 (0.5)	1.7 (0.3)	1.7 (0.3)
L-Arginine (μmol/l)	43 (4)	60 (8)	47 (7)	71* (13)	39 (5)	40 (6)
Total cholesterol (mmol/l)	5.9 (0.3)	5.6 (0.3)	6.1 (0.4)	5.8 (0.4)	5.8 (0.2)	6.3 (0.2)
Triglycerides (mmol/l)	1.4 (0.4)	1.4 (0.2)	1.7 (0.3)	2.2 (0.7)	1.5 (0.3)	1.9 (0.3)

Data are mean±S.E.M. *$P<0.05$ vs. baseline.

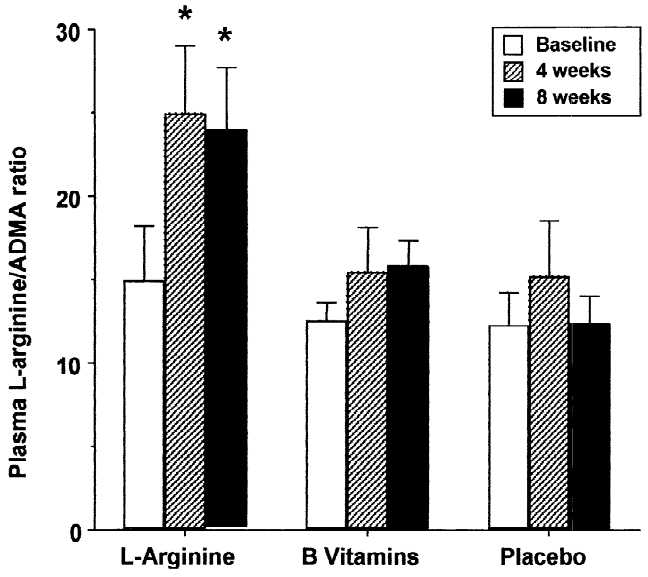

Fig. 3. Plasma L-arginine/ADMA ratio in patients with PAOD and hyperhomocyst(e)inemia. Data are mean±S.E.M. of $N=8$ patients per treatment group. *$P<0.05$ vs. baseline.

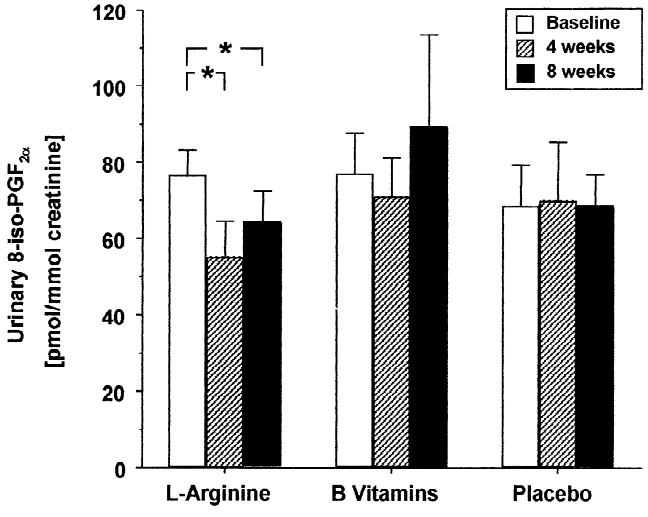

Fig. 4. Urinary excretion rate of 8-iso-$PGF_{2\alpha}$ in patients with PAOD and elevated homocyst(e)ine plasma concentrations. Data are mean±S.E.M. of $N=8$ patients per treatment group. *$P<0.05$ vs. baseline.

creatinine), but remained unchanged in the B vitamin and placebo groups (Fig. 4).

3.3. Functional status

None of the treatments had a significant effect on systemic blood pressure, systolic ankle blood pressure, or ankle-brachial pressure index (Table 2).

4. Discussion

The present data indicate that supplementation with folic acid and vitamins B-12 and B-6 during 8 weeks does not improve endothelium-dependent vasodilation in PAOD patients despite a significant reduction in plasma homocyst(e)ine concentration. By contrast, supplementation with L-arginine, which does not affect homocyst(e)ine plasma concentration but has been suggested to counteract the detrimental effects of elevated ADMA on endothelial function, significantly improved endothelium-dependent vasodilation. L-Arginine, but not B vitamins, also reduced urinary excretion of 8-iso-$PGF_{2\alpha}$, a non-invasive marker for oxidative stress in vivo.

High plasma homocyst(e)ine concentrations have been associated with increased risk of cardiovascular disease [2]. The implicit notion from these studies has been that lowering plasma homocyst(e)ine levels with pharmacotherapeutic measures may decrease the risk of cardiovascular disease, although several authors have emphasized the need for careful scrutiny of this inference in appropriate controlled clinical trials. Hyperhomocyst(e)inemia is due to vitamin deficiency in many healthy elderly subjects and in many PAOD patients [4]. An increased risk of cardiovascular disease progression may even be inferred from vitamin plasma levels within the normal range. In the present study baseline plasma concentrations of folic acid, vitamin B-12, and vitamin B-6 were above the lower limit of the recommended plasma concentration range [26]. Nonetheless, supplementation with these vitamins resulted in a significant reduction of

homocyst(e)ine levels, which is in accordance with previous data [7,26].

One mechanism by which homocysteine may affect physiological function of the vascular wall is reducing the biological activity of NO. The mechanism of this interaction between homocysteine and NO may be multifactorial [12–14]. Lentz et al. found that monkeys fed a diet enriched in methionine and deficient in folate and choline exhibited impaired endothelium-dependent vasodilation in vivo and ex vivo [9]. In two more recent studies, Tawakol et al. [10] and Woo et al. [11] found that humans with moderate hyperhomocyst(e)inemia also displayed impaired endothelium-dependent vasodilation. Flow-induced vasodilation in the brachial artery was significantly impaired in patients as compared to controls.

From these studies, it was tempting to speculate that lowering of plasma homocyst(e)ine levels by dietary supplementation with B vitamins may improve endothelial function. However, significant doubt has been shed on this concept by a recent study in nonhuman primates in which Lentz et al. [27] observed that dietary supplementation with B vitamins did not reverse a pre-existing dysfunction in endothelium-dependent vasodilation in monkeys with diet-induced hyperhomocyst(e)inemia and hypercholesterolemia. These investigators found no improvement of endothelium-dependent vasodilation in resistance vessels in vivo and in the carotid artery ex vivo, even after a period of 17 months of B vitamin supplementation in monkeys fed an atherogenic diet that produces both hyperhomocyst(e)inemia and hypercholesterolemia. Furthermore, the anticoagulant responses of thrombin infusion were also impaired and B vitamins failed to prevent intimal thickening in the carotid or iliac arteries [27]. Even in healthy human subjects with acute elevation of homocyst(e)ine by oral methionine loading, the synthesis of ADMA was stimulated due to a transmethylation reaction which occurs during formation of homocyst(e)ine from methionine [28]. After methionine bolus, elevation of homocyst(e)ine was associated with increased plasma concentration of ADMA, and reduced flow-mediated vasodilation. There was a significant inverse linear relationship between ADMA concentration and flow-mediated vasodilation, which was stronger than the relation between homocyst(e)ine concentration and flow-mediated vasodilation. The results are, however, in contrast to recent studies in humans [29,30]. Title et al. found an improvement of endothelium-dependent flow-mediated dilation in 25 patients with coronary artery disease after supplementation with folic acid for 4 months [29]. However, there was no positive effect of a combination with folic acid and antioxidants. Usui et al. showed that high dose folic acid supplementation improved endothelium-dependent vasodilation in humans after an acute methionine load [30]. In this study folic acid did not lower homocyst(e)ine concentration, which led these authors to conclude that the action of folic acid as a precursor for tetrahydrobiopterin, an essential cofactor of endothelial NO synthase, might be responsible for the beneficial effect on endothelium-dependent vasodilation [31,32]. Indeed, infusion of tetrahydrobiopterin has been shown to improve endothelial function in subjects with familial hypercholesterolemia in the absence of hyperhomocyst(e)inemia [32].

Our data show no improvement of flow-dependent vasodilation despite a significant reduction of homocyst(e)ine levels by about 50% after B vitamin treatment in patients with PAOD and hyperhomocyst(e)inemia. This observation was made despite the facts that (1) measurements were performed in the radial artery where no atherosclerotic plaques are present even in PAOD patients. Radial artery endothelium-dependent vasodilation is responsive to pharmacological intervention, as indicated by the improvement that can be obtained with vitamin C treatment [19] and with L-arginine as evidenced by the results of the present study; (2) dosage of B vitamins was sufficient and compliance of the patients was good, as assessed by individual plasma homocyst(e)ine and B vitamin levels after treatment; (3) the study had sufficient statistical power to detect even a small (2.5%) increase in endothelium-dependent vasodilation, as evidenced also by the significant improvement in the L-arginine group; and (4) the method of ultrasonic echo-tracking of the vascular wall that we applied is extremely sensitive and has an excellent reproducibility and variability as previously shown by two of us (BH, NA) [19]. Flow-dependent vasodilation determined by this method has been found to be largely dependent upon endothelial NO release, as indicated by the inhibitory effect of *N*-monomethyl-L-arginine (L-NMMA) [19].

The detrimental effect of homocysteine on endothelial function may be indirect in nature. We previously reported that plasma levels of the endogenous NO synthase inhibitor ADMA are increased in hyperhomocyst(e)inemic monkeys [14]. Recently, we have shown that a radioactively labeled methyl group from *S*-adenosylmethionine—an intermediate during the formation of homocysteine from methionine—is transferred to arginine to yield ADMA by protein arginine N-methyltransferases in cultured human endothelial cells [16]. Thus, endothelial dysfunction in hyperhomocyst(e)inemia may be secondary to elevated ADMA levels and reduced endothelial NO synthase activity (Fig. 5). We have recently identified ADMA as an independent cardiovascular risk factor in a prospective clinical trial [33]. Pursuing this hypothesis further, we investigated whether supplementation with L-arginine improves endothelial dysfunction in our patients. L-Arginine has previously been shown to reverse the detrimental effects of high ADMA levels on endothelial function in hypercholesterolemic subjects [23]. Indeed, L-arginine significantly improved endothelium-dependent vasodilation without affecting homocyst(e)ine levels. L-Arginine/

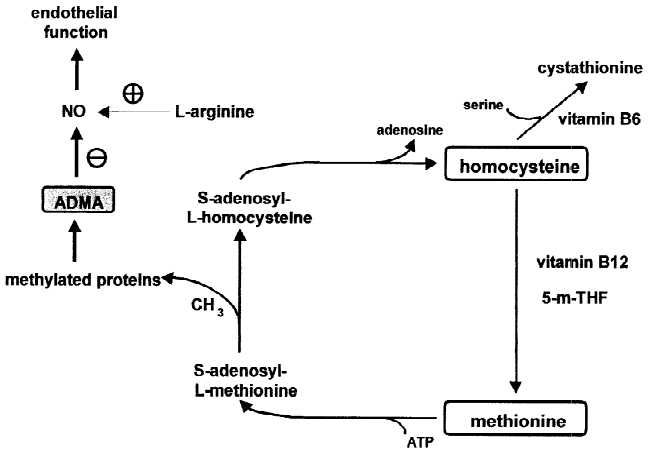

Fig. 5. Interaction between the L-arginine/NO and homocysteine pathway. Based on previous experimental findings we hypothesized that ADMA is formed due to the methylation of proteins. Methyl groups are derived from *S*-adenosyl-L-methionine, an intermediate in homocysteine metabolism. According to our hypothesis, lowering homocyst(e)ine plasma concentration with B vitamins should not decrease ADMA concentration. If homocyst(e)ine-induced endothelial dysfunction is mediated by elevation of ADMA, B vitamin treatment can be expected not to improve endothelial function. In contrast, exogenous supplementation with L-arginine, which does not affect homocyst(e)ine plasma concentration but increases the L-arginine/ADMA ratio and has been suggested to counteract the detrimental effects of elevated ADMA on NO synthase, significantly improved NO bioavailability and endothelial function. 5-m-THF, 5-methyl-tetrahydrofolate; ATP, adenosine triphosphate.

ADMA ratio was increased by about twofold during L-arginine supplementation, which may indicate enhanced substrate availability for NO synthase.

There is evidence that increased oxidative stress accounts for a significant proportion of endothelial dysfunction. Increased production of oxygen-derived free radicals such as superoxide anion has been linked to impaired endothelial vasomotor function in experimental models of atherosclerosis [34,35]. Recently, Heitzer et al. could show that endothelial dysfunction and increased vascular oxidative stress are predictors for the risk of cardiovascular events in patients with coronary artery disease [36]. Endothelial dysfunction in hyperhomocyst(e)inemia has been partly explained by increased oxidative stress [12,13]. Cardiovascular risk factors such as chronic cigarette smoking, diabetes mellitus and hypercholesterolemia have been associated with increased oxidative stress and lipid peroxidation (LPO). Measurement of isoprostanes can provide a sensitive and specific assessment of LPO in vitro and in vivo. 8-iso-prostaglandin $F_{2\alpha}$ is an abundant F_2-isoprostane which is excreted into the urine of humans [37]. The measurement of 8-iso-$PGF_{2\alpha}$ or of total F_2-isoprostanes in human urine has been established as a reliable method to non-invasively assess lipid peroxidation in vivo [38,39]. Different groups have consistently reported an increase in urinary 8-iso-$PGF_{2\alpha}$-excretion in different settings of increased cardiovascular risk like hypercholesterolemia, diabetes, smoking, and even in patients with acute myocardial infarction and unstable coronary disease [40–43]. We found that urinary excretion of 8-iso-$PGF_{2\alpha}$, a marker of lipid peroxidation in vivo, was increased in this population at baseline. Interestingly, B vitamin treatment did not significantly affect 8-iso-$PGF_{2\alpha}$ excretion, whereas L-arginine supplementation reduced this biomarker. Inhibition of NO synthase by ADMA increases vascular oxidative stress, which results in enhanced monocyte adhesion [44]. This effect is reversed by L-arginine and may explain the antioxidative actions of this amino acid that have previously been reported [45]. Kanani et al. showed that oxidative stress is involved in the endothelial dysfunction associated with acute hyperhomocyst(e)inemia after oral methionine loading [46]. We recently demonstrated that under the same conditions, ADMA levels are significantly increased in human subjects [28]. Taken together, this evidence strongly supports our hypothesis that enhanced production of ADMA underlies endothelial dysfunction in (acute and chronic) hyperhomocyst(e)inemia in humans, and that inhibition of endothelial NO synthase activity by ADMA causes oxidative stress which contributes to this endothelial dysfunction.

In conclusion, our study presents evidence for a lack of effect of homocyst(e)ine-lowering therapy with combined B vitamins on endothelium-dependent vasodilation in PAOD patients. There is strong evidence that the impact of homocysteine on endothelial function may be indirect in nature, due to elevation of the endogenous NO synthase inhibitor ADMA, and antagonized by supplementation with L-arginine. The results of randomized, large-scale clinical intervention trials need to be awaited and carefully scrutinized to definitely confirm or exclude a potential benefit of folic acid, vitamin B-12, and vitamin B-6 on incidence or progression of cardiovascular disease. Furthermore, other therapeutic interventions with lipid-lowering drugs, ACE inhibitors, and antioxidant agents seem to be an interesting tool to improve endothelial function in coronary and peripheral vessels in patients with hyperhomocyst(e)inemia.

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References

[1] Clarke R, Daly L, Robinson K et al. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149–1155.

[2] Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. J Am Med Assoc 1995;274:1049–1057.
[3] Perry IJ, Refsum H, Morris RW et al. Prospective study of serum total homocysteine concentration and risk of stroke in middle-aged British men. Lancet 1995;346:1395–1398.
[4] Malinow MR, Kang SS, Taylor LM et al. Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. Circulation 1989;79:1180–1188.
[5] Taylor LM, DeFrang RD, Harris Jr. EJ, Porter JM. The association of elevated plasma homocyst(e)ine with progression of peripheral arterial disease. J Vasc Surg 1991;13:128–136.
[6] Nygard O, Vollset SE, Refsum H, Brattström L, Ueland PM. Total homocysteine and cardiovascular disease. J Intern Med 1999;246:425–454, Review.
[7] Ubbink JB, Vermaak WJH, van der Merwe A et al. Vitamin requirements for the treatment of hyperhomocysteinemia in humans. J Nutr 1994;124:1927–1933.
[8] Lentz SR. Homocysteine and vascular dysfunction. Life Sci 1997;61:1205–1215.
[9] Lentz SR, Sobey CG, Piegors DJ et al. Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia. J Clin Invest 1996;98:24–29.
[10] Tawakol A, Omland T, Gerhard M, Nu JT, Creager MA. Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. Circulation 1997;95:1119–1121.
[11] Woo KS, Chook P, Lolin YI et al. Hyperhomocysteinemia is a risk factor for arterial endothelial dysfunction in humans. Circulation 1997;96:2542–2544.
[12] Starkebaum G, Harlan JM. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 1986;77:1370–1376.
[13] Lang D, Kredan MB, Moat SJ et al. Homocysteine-induced inhibition of endothelium-dependent relaxation in rabbit aorta: role for superoxide anions. Arterioscler Thromb Vasc Biol 2000;20:422–427.
[14] Böger RH, Bode-Böger SM, Sydow K, Heistad DD, Lentz SR. Plasma concentration of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, is elevated in monkeys with hyperhomocyst(e)inemia. Arterioscler Thromb Vasc Biol 2000;20:1557–1564.
[15] Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of NO synthesis in chronic renal failure. Lancet 1992;339:572–575.
[16] Böger RH, Sydow K, Borlak J et al. LDL cholesterol upregulates synthesis of asymmetric dimethylarginine (ADMA) in human endothelial cells. Involvement of *S*-adenosylmethionine-dependent methyltransferases. Circ Res 2000;87:99–105.
[17] Benzuly KH, Padgett RC, Kaul S et al. Functional improvement precedes structural regression of atherosclerosis. Circulation 1994;89:1810–1818.
[18] Hornig B, Kohler C, Drexler H. Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. Circulation 1997;95:1115–1118.
[19] Hornig B, Arakawa N, Kohler C, Drexler H. Vitamin C improves endothelial function of conduit arteries in patients with chronic heart failure. Circulation 1998;97:363–368.
[20] Ubbink JB, Hayward Vermaak WJ, Bissbort SH. Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441–446.
[21] Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. J Chromatogr 1987;422:43–52.
[22] Camp VM, Chipponi J, Faraj BA. Radioenzymatic assay for direct measurement of plasma pyridoxal 5′-phosphate. Clin Chem 1983;29:642–644.
[23] Böger RH, Bode-Böger SM, Szuba A et al. Asymmetric dimethylarginine: a novel risk factor for endothelial dysfunction. Its role in hypercholesterolemia. Circulation 1998;98:1842–1847.
[24] Tsikas D, Schwedhelm E, Fauler J et al. Specific and rapid quantification of 8-iso-prostaglandin $F_{2\alpha}$ in urine of healthy humans and patients with Zellweger syndrome by gas chromatography–tandem mass spectrometry. J Chromatogr B 1998;716:7–17.
[25] Böger RH, Bode-Böger SM, Thiele W et al. Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation 1997;95:2068–2074.
[26] Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ. Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia. Am J Clin Nutr 1993;57:47–53.
[27] Lentz SR, Piegors DJ, Malinow MR, Heistad DD. Supplementation of atherogenic diet with B vitamins does not prevent atherosclerosis or vascular dysfunction in monkeys. Circulation 2001;103:1006–1011.
[28] Böger RH, Lentz SR, Bode-Böger SM, Knapp HR, Haynes WG. Elevation of asymmetrical dimethylarginine may mediate endothelial dysfunction during experimental hyperhomocyst(e)inemia in humans. Clin Sci 2001;100:161–167.
[29] Title LM, Cummings PM, Giddens K, Genest Jr. JJ, Nassar BA. Effect of folic acid and antioxidant vitamins on endothelial dysfunction in patients with coronary artery disease. J Am Coll Cardiol 2000;36:758–765.
[30] Usui M, Matsuoka H, Miyazaki H et al. Endothelial dysfunction by acute hyperhomocyst(e)inaemia: restoration by folic acid. Clin Sci 1999;96:235–239.
[31] Pollock JS, Förstermann U, Mitchell JA et al. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc Natl Acad Sci USA 1991;88:10480–10484.
[32] Stroes E, Kastelein J, Cosentino F et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest 1997;99:41–46.
[33] Zoccali C, Bode-Böger SM, Mallamaci F et al. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet 2001;358:2113–2117.
[34] Keaney Jr. JF, Vita JA. Atherosclerosis, oxidative stress, and antioxidant protection in endothelium-derived relaxing factor action. Prog Cardiovasc Dis 1995;38:129–154.
[35] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840–844.
[36] Heitzer T, Schlinzig T, Krohn K, Meinertz T, Münzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 2001;104:2673–2678.
[37] Morrow JD, Minton TA, Badr KF, Roberts 2nd LJ. Evidence that the F2-isoprostane, 8-epi-prostaglandin F2 alpha, is formed in vivo. Biochim Biophys Acta 1994;1210:244–248.
[38] Delanty N, Reilly M, Pratico D et al. 8-Epi PGF2 alpha: specific analysis of an isoeicosanoid as an index of oxidant stress in vivo. Br J Clin Pharmacol 1996;42:15–19.
[39] Lawson JA, Rokach J, FitzGerald GA. Isoprostanes: formation, analysis and use as indices of lipid peroxidation in vivo. J Biol Chem 1999;274:24441–24444.
[40] Morrow JD, Frei B, Longmire AV et al. Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. Smoking as a cause of oxidative damage. N Engl J Med 1995;332:1198–1203.
[41] Davi G, Ciabattoni G, Concoli A et al. In vivo formation of 8-iso-prostaglandin $F_{2\alpha}$ and platelet activation in diabetes mellitus. Effects of improved metabolic control and vitamin E supplementation. Circulation 1999;99:224–229.
[42] Reilly MP, Practico D, Delanty N et al. Increased generation of distinct F_2-isoprostanes in hypercholesterolemia. Circulation 1998;98:2822–2828.

[43] Reilly MP, Delanty N, Roy L et al. Increased formation of the isoprostanes $IPF_{2\alpha}$-I and 8-iso$PGF_{2\alpha}$ in acute coronary angioplasty: evidence for oxidant stress during coronary reperfusion in humans. Circulation 1997;96:3314–3320.

[44] Chan JR, Böger RH, Bode-Böger SM et al. Asymmetric dimethylarginine increases mononuclear cell adhesiveness in hypercholesterolemic humans. Arterioscler Thromb Vasc Biol 2000;20:1040–1046.

[45] Böger RH, Bode-Böger SM, Mügge A et al. Supplementation of hypercholesterolaemic rabbits with L-arginine reduces the vascular release of superoxide anions and restores NO production. Atherosclerosis 1995;117:273–284.

[46] Kanani PM, Sinkey CA, Browning RL et al. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. Circulation 1999;100:1161–1168.