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No Evidence for Oxidative Stress as a Mechanism of Action of Hyperhomocysteinemia in Humans

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Oxidative stress has been suggested as one of the physiopathologic conditions underlying the association of total plasma homocysteine (p-tHcy) with cardiovascular disease (CVD), but this hypothesis has not been validated in human epidemiological studies. We measured plasma and erythrocyte antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD), along with serum lipid-soluble antioxidants alpha-tocopherol, beta-carotene, lycopene and retinol, in a sample of 123 healthy elderly subjects (54 men, 69 women). Plasma malondialdehyde (p-MDA) was determined as a marker of lipid peroxidation, and p-tHcy was quantified by HPLC. No significant differences were found for p-MDA, GPx or SOD activities or serum antioxidant concentrations, in subjects with elevated p-tHcy ($\geq 15\,\mu$mol/l) as compared to those with lower plasma homocysteine. Hyperhomocysteinemia did not lead to increased risk of having the highest p-MDA values, in either sex. We found no evidence that p-tHcy was associated with lipid peroxidation in this elderly human sample. Our results do not support the view that hyperhomocysteinemia would induce an adaptive response of antioxidant systems, either. More epidemiologic and clinical research is needed to clarify whether homocysteine promotes atherosclerosis by means of an oxidative stress mechanism.

Keywords: Homocysteine; Lipid peroxidation; Antioxidant enzymes; Antioxidant vitamins; Elderly

INTRODUCTION

Thirty five years after McCully first hypothesized that homocysteine promoted atherosclerosis,[1] it is now widely accepted that total plasma homocysteine (p-tHcy) concentration predicts cardiovascular disease (CVD) risk, independently of other known risk factors,[2–5] and in a dose-response manner[6]. Nevertheless, although there have been great efforts to elucidate the patho-physiologic mechanism by which p-tHcy could lead to increased risk of CVD, it remains elusive, and the question of whether p-tHcy is a causal factor or just an incidental marker of vascular dysfunction has yet to be answered.

Although elevated p-tHcy has been connected to vascular disorders by several different mechanisms,[7] the hypothesis that homocysteine-induced oxidative stress could account for the negative effects of this amino acid on vascular physiology was postulated because of its biological plausibility.[8] Homocysteine is an aminothiol susceptible of autooxidation and subsequent generation of reactive oxygen species, such as superoxide anions and hydrogen peroxide.[9,10] Besides, it has been shown to inhibit the activity of the antioxidant enzyme glutahione peroxidase (GPx; EC 1.11.1.9), a situation that would aggravate the imbalance in favor of an oxidative status.[11,12] *In vitro* and *in vivo* studies suggest that p-tHcy impairs endothelial function by altering its vasodilator and anticoagulant responses,[13–15] and by decreasing the availability of nitric oxide (NO).[16,17] This decline in available NO is explained either through its reaction with homocysteine-induced superoxide radicals to form peroxynitrite[8,18] or by inhibition of GPx, thus favoring a prooxidant environment that would

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promote the oxidation, and inactivation, of NO.[12] Furthermore, it was found that these changes in endothelial function due to p-tHcy exposure are prevented by administering antioxidants, such as vitamins C and E.[14,19] In the light of these findings, p-tHcy was postulated as the first risk factor to induce atherosclerosis by means of an oxidative stress mechanism.[8]

Nevertheless, in spite of the plausibility of the hypothesis, epidemiological research failed to find an association between p-tHcy and indices of pro- or antioxidant status in human populations.[20–23] While this fact does not necessarily invalidate the hypothesis, because of limitations of epidemiological studies, it does represent a serious drawback to accepting it.

To further investigate the homocysteine-induced oxidative stress hypothesis, in this cross-sectional study, we analyzed whether physiological concentrations of p-tHcy were associated either with plasma antioxidant status or with oxidative damage. The antioxidant status was evaluated by measuring the activities of GPx and superoxide dismutase (SOD; EC 1.15.1.1) and the serum concentrations of non-enzymatic antioxidants, such as alpha-tocopherol, beta-carotene, lycopene and retinol. Plasma malondialdehyde (p-MDA) was used as a marker of lipid peroxidation, as it is the most abundant individual aldehyde generated by free radical attack on polyunsaturated fatty acids of cell membranes.

This analysis is part of an ongoing prospective study on diet and disease carried out in a healthy institutionalized population aged 60 years or older.

SUBJECTS AND METHODS

Sample Characteristics

The final sample analysed consisted of 123 apparently healthy subjects (54 men and 69 women) between 60 and 82 years old. A total of 160 participants were recruited from seven nursing homes distributed throughout Asturias, a Northern Spanish region. No subject with history of cancer, or suffering from CVD was included in the study, as these were exclusion criteria for our ongoing prospective investigation. For the present analysis, subjects on antiepileptic, antidepressant, antiinflammatory or thyroid hormone therapy were also excluded, since these drugs could affect either homocysteine or malondialdehyde (MDA) plasma concentration.[24–26] On this basis, a total of 37 subjects were excluded from the analysis. None of the participants was taking antioxidant supplements.

Previously to data collection, participants were informed about the nature, methodology and objectives of the study in order to obtain their consent. The study protocol was approved by the Committee on Ethical Research of the Oviedo University Hospital.

Data Collection

Height of the participants was obtained to the nearest 1 mm with a stadiometer (Año-Sayol, Barcelona, Spain) by having the subjects barefoot, in an upright position. Weight was measured with a 500 g precision scale (Seca, Hamburg, Germany) in light clothes. Quetelet's body mass index was calculated as weight (in kg) divided by square of the height (in m^2) for each subject.

Information about habit of smoking and alcohol consumption (type and amount) was registered by means of an interview.

Blood Measurements

Fasting blood was collected by venipuncture and kept cold in dark until processed in the laboratory. Samples were centrifuged within the next 2 h following the extraction for plasma while, for serum, blood was allowed to clot in dark at room temperature, and then centrifuged at 3500 rpm for 15 min. Erythrocytes were washed three times in cold saline solution (9.0 g/l NaCl) and hemolyzed by adding distilled water containing 5 ml/l of Triton X-100). Aliquots were stored at −70°C until biochemical analyses were performed. Membrane-free hemolysate was obtained by centrifugation and hemoglobin concentration was assayed by the cyanometahemoglobin method (Sigma cat.no. 541-2, Sigma Chemical Co., USA).

Total plasma homocysteine was determined by reverse-phase HPLC (Biorad) with fluorimetric detection,[27] together with cysteine and cysteine-glycine as controls to assure the analytical stability of the procedure, by injecting a volume of 20 μl of sample at a flow rate of 0.8 ml/min. Measurements were performed in the Laboratory of Clinical Analysis of the Asturias Central Hospital (Oviedo, Spain).

MDA concentration was measured in plasma with the commercial kit LPO-586 (Byoxytech, Oxis International S.A., France). Samples were deproteinized by adding 65 μl of trichloroacetic acid to 450 μl of plasma (final acid concentration of ~10%) and centrifuged 10 min at 13000 rpm. A measure of 200 μl of supernatant were assayed for MDA, in the presence of hydrochloric acid to prevent interference of 4-hydroxynonenal, with spectrophotometric detection at 586 nm.

Antioxidant enzyme activities of GPx and SOD were determined both in plasma and red blood cells. Plasma and erythrocyte SOD were assayed with the SOD-525 kit, and red blood cell GPx, with the

GPx-340 test, while plasma GPx was immunoenzymatically determined with the pl-Gpx-EIA kit. All commercial kits for measurement of antioxidant activities were obtained from Byoxytech (Oxis International).

Serum α-tocopherol, β-carotene, lycopene and retinol concentrations were quantified by HPLC on an Alliance equipment (Waters). Prior to analyses, protein was removed from the samples by precipitation with ethanol. From the supernatant, 400 µl were evaporated in a Speed-Vac-Concentrator at room temperature and the residue was resuspended in 100 µl of ethanol/dioxane (1:1), before adding 150 µl of acetonitrile. Extracted samples (100 µl) were injected and eluted in isocratic conditions with 5% water and 95% of a mixture of acetonitrile/tetrahydrofuran/methanol/water (684:220:68:28) at a flow rate of 1.5 ml/min. Maximum absorbance for each vitamin was 293 for α-tocopherol, 456 nm for β-carotene, 474 nm for lycopene and 325 nm for retinol. Quantification was performed by area integration in chromatograms. Vitamin standards used for peak identification and quantification were purchased from Sigma (Sigma Chemical Co.).

Plasma total cholesterol and triglycerides were determined by standard enzymatic methods.

The concentrations of α-tocopherol, β-carotene, lycopene and retinol were adjusted for plasma lipids according to the formula:[28]

Antioxidant [molar concentration] × (5.6 mmol/l cholesterol + 1.24 mmol/triglycerides)/(actual cholesterol + triglycerides levels in individual plasma).

Statistical Analyses

Kolmogorov-Smirnov tests were performed to check that the distribution of variables followed a gaussian pattern. When variables were not normally distributed or the variance of errors was not constant across factor levels (i.e. heteroskedasticity), variables were transformed by taking the natural logarithm before performing statistical comparisons. Differences in continuous variables by sex were analysed by Student's *t*-tests. The Chi-square test was used to check for differences in the percentage of smokers between men and women.

Logistic regression models were conducted in order to estimate the odds ratio (OR) of being in the highest tertile of p-MDA for subjects with elevated p-tHcy (≥15 µmol/l, as defined by Kang *et al.*[29]) as compared to those with lower p-tHcy concentration. The multivariate model included age, Quetelet's body mass index, habit of smoking and ethanol consumption as covariates with potential confounding effect on p-MDA.

A simple linear regression analysis was conducted to predict p-MDA with p-tHcy as a continuous independent variable, and a scatter plot with a superimposed regression line was used to represent the data.

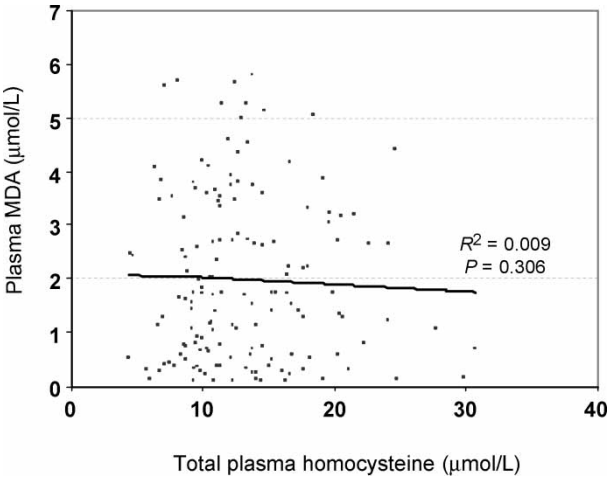

FIGURE 1 Scatterplot of plasma malondialdehyde (p-MDA) against total plasma homocysteine concentration ($n = 123$).

The SPSS v.11.0 package for Windows was used for statistical analyses (SPSS Inc., Chicago, IL., USA), and Microsoft® Excel 2000 software (Microsoft Corp., Redmond, WA., USA) for graphical representation of Fig. 1. Differences were considered significant at $P < 0.05$ level. All *P*-values are two-tailed.

RESULTS

Table I shows mean values of p-tHcy, p-MDA, enzymatic and lipid-soluble serum antioxidants, as well as age, BMI and life-style factors related to oxidative stress, by sex. Mean age was 73.2 years for men, and 74.2 years for women. Since men had a significantly higher p-tHcy concentration, analyses were performed separately by sex. No significant differences were observed for p-MDA, SOD, GPx or lipid-soluble antioxidants by sex. On average, men were more prone to smoke and consumed more alcohol than women did.

Blood concentrations of MDA, enzymes and lipid-soluble antioxidants are presented in Table II according to p-tHcy levels and sex. Men with p-tHcy below 15 µmol/l showed slightly higher red blood cell GPx activity, although not significant ($P = 0.067$). No statistically significant differences were observed for any of the considered variables, with respect to homocysteine levels.

To estimate whether subjects with elevated p-tHcy concentration (≥15 µmol/l) were at higher risk for being in the highest tertile of p-MDA (using sex-specific cutoffs), a logistic regression analysis was conducted for men and women separately. The two lowest tertiles of p-MDA were considered together as the reference group. Results shown in Table III

TABLE I Characteristics of the study sample by sex

	Men (n=54)	Women (n=69)
Age (years)	73.2 ± 5.3*	74.2 ± 4.5
Body mass index (Kg/m^2)	27.5 ± 4.4	28.6 ± 5.2
Plasma total homocysteine (μmol/l)	13.9 ± 6.2	12.7 ± 4.1*
Hyperhomocysteinemia, % (n)	33.3 (18)	26.1 (18)
Plasma MDA (μmol/l)	1.8 ± 1.4	2.0 ± 1.7
Plasma GPx (U/l)	113.1 ± 98.3	125.3 ± 105.5
Red blood cell GPx (U/g Hb)	3.2 ± 1.3	3.6 ± 1.6
Plasma SOD (kU/l)	34.1 ± 9.7	34.4 ± 10.8
Red blood cell SOD (kU/g Hb)	23.4 ± 7.1	25.0 ± 8.9
Serum alpha-tocopherol (μmol/L)†	19.9 ± 8.9	23.3 ± 8.3
Serum beta-carotene (nmol/l)†	150.9 ± 137.8	182.7 ± 156.6
Serum lycopene (nmol/l)†	199.8 ± 177.3	175.0 ± 109.9
Serum retinol (μmol/l)†	1.29 ± 0.49	1.27 ± 0.38
Smoker (%)	22.2	4.3*
Cigarettes/day‡	13.5 ± 9.2	3.5 ± 3.0
Ethanol consumption (g/day)¶	16.8 ± 15.9	4.9 ± 4.9*

* Values represent mean ± standard deviation. † Adjusted for plasma lipids. ‡ Only smokers. ¶ Only alcohol drinkers. *$P < 0.05$.

indicate that, after adjusting for potential confounders (age, BMI, smoking and consumption of alcohol), a high homocysteine concentration was not associated with an increased risk of high p-MDA levels, either in men or in women.

P-MDA was plotted against p-tHcy concentration for the whole sample (Fig. 1). The regression line that best fits the data had a flat slope, while the model did not account for a sizeable amount of p-MDA variance ($R^2 = 0.009$).

DISCUSSION

To our best knowledge, this is the first report to analyze the association of p-tHcy with oxidant status in a sample of healthy elderly, taking into account several indexes of the oxidant/antioxidant balance. Even though our study represents a more comprehensive evaluation than that of other previous studies,[20,30] our data do not support the hypothesis that p-tHcy is associated with oxidative stress.

The mechanism by which p-tHcy increases CVD risk has not been elucidated yet. The oxidative stress hypothesis would provide a plausible theoretical basis for this association. Homocysteine has been proposed to promote an oxidative environment by two different, yet not exclusive, mechanisms: (a) by directly generating or inducing reactive oxygen species, and (b) by counteracting antioxidant defenses. Our data do not support any of these hypotheses.

In regard to the first mechanism, we have found that lipid peroxidation does not increase as p-tHcy concentration does (Fig. 1); the same lack of association has been found by other epidemiological researches.[21–23,30] In fact, most of the data that support the homocysteine-induced oxidative stress hypothesis come from *in vitro* studies or from animal *in vivo* investigations where oxidative parameters are measured following a methionine load.[7,31,32] As pointed out by Jacobsen,[33] *in vitro* studies have been performed by administering supraphysiological doses, up to 5000 μM of homocysteine,[34] in such conditions (absence of *in vivo* antioxidant systems) that generation of free radicals scarcely reflects the cellular physiological environment. In line with this, the methionine load test consists of the administration of a high dose of methionine (100 mg/kg of body weight), which, in humans, has been shown to increase plasma homocysteine up to levels considered to represent hyperhomocysteinemia.[7] In the light of our results, we consider that evidence from these trials has to be evaluated with caution, as the transient hyperhomocysteinemia induced might not be a valid physiological model to simulate the long term hyperhomocysteinemic condition of an organism in which, in all likelihood, adaptive responses have evolved that handle this stressful situation.

There exists controversy on whether homocysteine affects the activity of antioxidant enzymes. Some authors have found that GPx is inhibited by free

TABLE II Plasma malondialdehyde, antioxidant enzymes (plasma and red blood cell) and serum lipid-soluble antioxidants in regard to hyperhomocysteinemia, by sex

	Men		Women	
	p-tHcy < 15 μmol/l (n=36)	p-tHcy ≥ 15 μmol/l (n=18)	p-tHcy < 15 μmol/l (n=51)	p-tHcy ≥ 15 μmol/l (n=18)
Plasma MDA (μmol/l)	1.80 ± 1.45*	1.74 ± 1.34	2.26 ± 1.77	1.44 ± 1.26
Plasma GPx (U/l)	104.6 ± 104.4	130.1 ± 86.3	131.6 ± 106.6	107.3 ± 102.9
Red blood cell GPx (U/g Hb)	3.39 ± 1.27	2.67 ± 1.4	3.54 ± 1.49	3.62 ± 2.04
Plasma SOD (kU/l)	33.5 ± 10.0	33.3 ± 9.3	33.3 ± 8.5	37.5 ± 15.2
Red blood cell SOD (kU/g Hb)	24.0 ± 6.7	22.0 ± 7.8	25.0 ± 9.5	24.8 ± 6.9
Serum alpha-tocopherol (μmol/l)†	19.0 ± 10.4	19.2 ± 8.2	21.9 ± 8.0	24.1 ± 7.9
Serum beta-carotene (nmol/l)†	154.4 ± 125.6	143.8 ± 163.6	177.5 ± 163.3	197.6 ± 138.6
Serum lycopene (nmol/l)†	204.8 ± 179.0	189.7 ± 178.3	177.1 ± 112.2	169.1 ± 105.7
Serum retinol (μmol/l)†	1.28 ± 0.41	1.32 ± 0.63	1.19 ± 0.33	1.32 ± 0.40

* Values are age-adjusted mean ± standard deviation. † Adjusted for plasma lipids.

TABLE III Odds ratio (OR) of being in the upper tertile of plasma malondialdehyde (p-MDA)* for subjects with elevated homocysteine, by sex

	Non-adjusted model		Multivariate model†	
	OR	(95% CI)	OR	(95% CI)
Men ($n = 54$)				
p-tHcy $< 15\,\mu$mol/l	1	–	1	–
p-tHcy $\geq 15\,\mu$mol/l	1.27	(0.39–4.12)	2.94	(0.66–13.09)
Women ($n = 69$)				
p-tHcy $< 15\,\mu$mol/l	1	–	1	–
p-tHcy $\geq 15\,\mu$mol/l	0.65	(0.20–2.10)	0.84	(0.22–3.17)

*High tertile cutoff for p-MDA: men $> 2.22\,\mu$mol/l, women $> 2.65\,\mu$mol/l. †Covariates: age (years), Quetelet's body mass index (kg/m^2), smoking habit (0 = no, 1 = yes), ethanol consumption (g/day).

homocysteine either at physiological or supraphysiological concentrations.[11,12] Chen *et al.*[11] propose that GPx is protective against CVD, and thus, the direct association between homocysteine and vascular disease might, in fact, indirectly reflect the fall in GPx activity. In our study, men (but not women) with higher p-tHcy have slightly lower red blood cell GPx activity ($P = 0.067$), but no differences were observed for any other antioxidant activity measured, either in men or women. Supporting these findings, Moat *et al.*[22] have recently carried out and intervention to lower p-tHcy through folate-enriched diet or folic acid tablets and found that while p-tHcy significantly decreased, there were no statistically significant differences in plasma/erythrocyte GPx or SOD activities between supplemented subjects and control groups. Interestingly, and according to our results, plasma MDA concentration did not vary across groups (control or supplemented) with the intervention. But the question remains unclear as the same authors had found in a previous study an adaptive increase in the activity of both GPx and SOD in subjects with p-tHcy $>20\,\mu$mol/l.[35]

Non-enzymatic plasma antioxidants such as α-tocopherol, β-carotene, lycopene and retinol, play an important role in balancing the oxidant/antioxidant status.[36] The fact that we were unable to find differences in these parameters between subjects with higher and those with lower p-tHcy values allows us to discard the possibility that the non-enzymatic antioxidant status of hyperhomocysteinemic subjects might underlie the lack of association between p-tHcy and p-MDA in the present study.

Homocysteine is a thiol compound and, thus, capable of auto-oxidizing to produce superoxide radical anions. But, under physiological conditions, only 2% exists in the reduced form, the rest consisting of protein-bound homocysteine, and non protein-bound disulfides (homocystine and mixed disulfides).[7,33] Because of that, the relevance of the auto-oxidative mechanism for generating free radical species has to be reevaluated, moreover when taking into account that total plasma cysteine concentration exceeds that of p-tHcy by 25–30 times, and that 5–6% of that total amount exists in the reduced form;[33] so homocysteine only represents a small proportion of the total pool of plasma thiols. This fact, along with the results of the present study and other epidemiological investigations do not point in the direction that oxidative stress would account for the harming effect of homocysteine on cardiovascular health.

In interpreting these results some considerations should be kept in mind. First, the study has been carried out in a relatively small number of participants which we recognize as a limitation, but, on the other hand, the analysis of multiple variables to assess antioxidant/oxidant status is its main strength. Second, results are taken from cross-sectional analyses so they cannot be used to imply a causal relationship. Also, the measure of the degree of oxidative stress that a subject is undergoing should be mentioned. No standardized method has been accepted as measuring the *in vivo* oxidative stress status of humans. HPLC-based methods remain the gold standard for the analysis of lipid peroxidation products because of their high chemical specificity, but these methods take much longer and are more expensive, thus limiting their use in epidemiological research. Other studies in which p-MDA was analyzed by HPLC do report similar mean values to those reported here.[37] Finally, our study has been carried out on healthy elderly with normal-to-mild homocysteine levels, so results cannot be extrapolated to predict how higher concentrations of p-tHcy would affect lipid peroxidation.

In conclusion, while our results (and those of other studies[20–23,38,39]) do not support an effect of homocysteine on oxidant/antioxidant status, others do,[40,41] and some others find the association to be dependent of the age and sex of the sample.[30] Whereas there is no solid proof in humans, scientific researchers should consider that the homocysteine-induced oxidative stress hypothesis is, though plausible, a possibility for which the evidence is unconvincing. Recent research

suggests additional plausible mechanisms by which elevated p-tHcy could be connected to vascular disorders: it has been shown to increase monocyte and T-cell adhesion to human aortic endothelial cells,[42] and to cause dysregulation of cholesterol metabolism by enhancing the expression of genes responsible for cholesterol/triglyceride biosynthesis and uptake.[43,44] Our results suggest that oxidative stress and homocysteinemia act as independent cardiovascular risk factors.

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References

[1] McCully, K.S. (1969) "Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis", *Am. J. Pathol.* **56**, 111–128.
[2] Eikelboom, J.W., Lonn, E., Genest, J., Jr., Hankey, G. and Yusuf, S. (1999) "Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence", *Ann. Intern. Med.* **131**, 363–375.
[3] The Homocysteine Studies Collaboration (2002) "Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis", *JAMA* **288**, 2015–2022.
[4] Vollset, S.E., Refsum, H., Tverdal, A., Nygård, O., Nordrehaug, J.E., Tell, G.S. and Ueland, P.M. (2001) "Plasma total homocysteine and cardiovascular and noncardiovascular mortality: the HORDALAND homocysteine study", *Am. J. Clin. Nutr.* **74**, 130–136.
[5] Wald, N.J., Watt, H.C., Law, M.R., Weir, D.G., McPartlin, J. and Scott, J.M. (1998) "Homocysteine and ischemic heart disease: results of a prospective study with implications regarding prevention", *Arch. Intern. Med.* **158**, 862–867.
[6] Boushey, C.J., Beresford, S.A., Omenn, G.S. and Motulsky, A.G. (1995) "A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes", *JAMA* **274**, 1049–1057.
[7] Durand, P., Prost, M., Loreau, N., Lussier-Cacan, S. and Blache, D. (2001) "Impaired homocysteine metabolism and atherothrombotic disease", *Lab. Investig.* **81**, 645–672.
[8] Loscalzo, J. (1996) "The oxidant stress of hyperhomocyst(e)inemia", *J. Clin. Investig.* **98**, 5–7.
[9] Heinecke, J.W., Rosen, H., Suzuki, L.A. and Chait, A. (1987) "The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells", *J. Biol. Chem.* **262**, 10098–10103.
[10] Misra, H.P. (1974) "Generation of superoxide free radical during the autoxidation of thiols", *J. Biol. Chem.* **249**, 2151–2155.
[11] Chen, N., Liu, Y., Greiner, C.D. and Holtzman, J.L. (2000) "Physiologic concentrations of homocysteine inhibit the human plasma GSH peroxidase that reduces organic hydroperoxides", *J. Lab. Clin. Med.* **136**, 58–65.
[12] Upchurch, G.R., Jr., Welch, G.N., Fabian, A.J., Freedman, J.E., Johnson, J.L., Keaney, J.F., Jr. and Loscalzo, J. (1997) "Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase", *J. Biol. Chem.* **272**, 17012–17017.
[13] Lentz, S.R., Sobey, C.G., Piegors, D.J., Bhopatkar, M.Y., Faraci, F.M., Malinow, M.R. and Heistad, D.D. (1996) "Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia", *J. Clin. Investig.* **98**, 24–29.
[14] Nappo, F., de Rosa, N., Marfella, R., de Lucia, D., Ingrosso, D., Perna, A.F., Farzati, B. and Giugliano, D. (1999) "Impairment of endothelial functions by acute hyperhomocysteinemia and reversal by antioxidant vitamins", *JAMA* **281**, 2113–2118.
[15] Woo, K.S., Chook, P., Lolin, Y.I., Cheung, A.S., Chan, L.T., Sun, Y.Y., Sanderson, J.E., Metreweli, C. and Celermajer, D.S. (1997) "Hyperhomocyst(e)inemia is a risk factor for arterial endothelial dysfunction in humans", *Circulation* **96**, 2542–2544.
[16] Mujumdar, V.S., Aru, G.M. and Tyagi, S.C. (2001) "Induction of oxidative stress by homocyst(e)ine impairs endothelial function", *J. Cell Biochem.* **82**, 491–500.
[17] Stamler, J.S., Osborne, J.A., Jaraki, O., Rabbani, L.E., Mullins, M., Singel, D. and Loscalzo, J. (1993) "Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen", *J. Clin. Investig.* **91**, 308–318.
[18] Lang, D., Kredan, M.B., Moat, S.J., Hussain, S.A., Powell, C.A., Bellamy, M.F., Powers, H.J. and Lewis, M.J. (2000) "Homocysteine-induced inhibition of endothelium-dependent relaxation in rabbit aorta: role for superoxide anions", *Arterioscler. Thromb. Vasc. Biol.* **20**, 422–427.
[19] Chambers, J.C., McGregor, A., Jean-Marie, J., Obeid, O.A. and Kooner, J.S. (1999) "Demonstration of rapid onset vascular endothelial dysfunction after hyperhomocysteinemia: an effect reversible with vitamin C therapy", *Circulation* **99**, 1156–1160.
[20] Blom, H.J., Engelen, D.P., Boers, G.H., Stadhouders, A.M., Sengers, R.C., de Abreu, R., TePoele-Pothoff, M.T. and Trijbels, J.M. (1992) "Lipid peroxidation in homocysteinaemia", *J. Inherit. Metab. Dis.* **15**, 419–422.
[21] Carluccio, F., Siems, W., Stefanelli, G., Sommerburg, O., Grune, T., Riedel, E. and Hampl, H. (2002) "Homocysteine in chronic renal failure in relation to renal anemia and to oxidative stress parameters 4-hydroxynonenal and malondialdehyde", *Clin. Nephrol.* **58**(Suppl. 1), S26–S30.
[22] Moat, S.J., Hill, M.H., McDowell, I.F., Pullin, C.H., Ashfield-Watt, P.A., Clark, Z.E., Whiting, J.M., Newcombe, R.G., Lewis, M.J. and Powers, H.J. (2003) "Reduction in plasma total homocysteine through increasing folate intake in healthy individuals is not associated with changes in measures of antioxidant activity or oxidant damage", *Eur. J. Clin. Nutr.* **57**, 483–489.
[23] Sebekova, K., Krajcovicova-Kudlackova, M., Blazicek, P., Parrak, V., Schinzel, R. and Heidland, A. (2003) "Functional hyperhomocysteinemia in healthy vegetarians: no association with advanced glycation end products, markers of protein oxidation, or lipid peroxidation after correction with vitamin B(12)", *Clin. Chem.* **49**, 983–986.
[24] Bilici, M., Efe, H., Koroglu, M.A., Uydu, H.A., Bekaroglu, M. and Deger, O. (2001) "Antioxidative enzyme activities and lipid peroxidation in major depression: alterations by antidepressant treatments", *J. Affect. Disord.* **64**, 43–51.
[25] Catargi, B., Parrot-Roulaud, F., Cochet, C., Ducassou, D., Roger, P. and Tabarin, A. (1999) "Homocysteine, hypothyroidism, and effect of thyroid hormone replacement", *Thyroid* **9**, 1163–1166.
[26] Varela-Moreiras, G. (2001) "Nutritional regulation of homocysteine: effects of drugs", *Biomed. Pharmacother.* **55**, 448–453.
[27] Araki, A. and Sako, Y. (1987) "Determination of free and total homocysteine in human plasma by high- performance liquid chromatography with fluorescence detection", *J. Chromatogr.* **422**, 43–52.
[28] Panemangalore, M. and Lee, C.J. (1992) "Evaluation of the indices of retinol and alpha-tocopherol status in free-living elderly", *J. Gerontol.* **47**, B98–B104.
[29] Kang, S.S., Wong, P.W. and Malinow, M.R. (1992) "Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease", *Annu. Rev. Nutr.* **12**, 279–298.
[30] Powers, R.W., Majors, A.K., Lykins, D.L., Sims, C.J., Lain, K.Y. and Roberts, J.M. (2002) "Plasma homocysteine and malondialdehyde are correlated in an age- and gender-specific manner", *Metabolism* **51**, 1433–1438.

[31] Jara-Prado, A., Ortega-Vázquez, A., Martínez-Ruano, L., Ríos, C. and Santamaría, A. (2003) "Homocysteine-induced brain lipid peroxidation: effects of NMDA receptor blockade, antioxidant treatment, and nitric oxide synthase inhibition", *Neurotox. Res.* **5**, 237–243.
[32] Streck, E.L., Vieira, P.S., Wannmacher, C.M., Dutra-Filho, C.S., Wajner, M. and Wyse, A.T. (2003) "*In vitro* effect of homocysteine on some parameters of oxidative stress in rat hippocampus", *Metab. Brain. Dis.* **18**, 147–154.
[33] Jacobsen, D.W. (2000) "Hyperhomocysteinemia and oxidative stress: time for a reality check?", *Arterioscler.Thromb. Vasc. Biol.* **20**, 1182–1184.
[34] Heydrick, S.J., Weiss, N., Thomas, S.R., Cap, A.P., Pimentel, D.R., Loscalzo, J. and Keaney, J.F., Jr. (2004) "L-homocysteine and L-homocystine stereospecifically induce endothelial nitric oxide synthase-dependent lipid peroxidation in endothelial cells", *Free Radic. Biol. Med.* **36**, 632–640.
[35] Moat, S.J., Bonham, J.R., Cragg, R.A. and Powers, H.J. (2000) "Elevated plasma homocysteine elicits an increase in antioxidant enzyme activity", *Free Radic. Res.* **32**, 171–179.
[36] Lasheras, C., Huerta, J.M., González, S., Braña, A.F., Patterson, A.M. and Fernández, S. (2002) "Independent and interactive association of blood antioxidants and oxidative damage in elderly people", *Free Radic. Res.* **36**, 875–882.
[37] Steghens, J.P., van Kappel, A.L., Denis, I. and Collombel, C. (2001) "Diaminonaphtalene, a new highly specific reagent for HPLC-UV measurement of total and free malondialdehyde in human plasma or serum", *Free Radic. Biol. Med.* **31**, 242–249.
[38] Chao, C.L., Kuo, T.L. and Lee, Y.T. (2000) "Effects of methionine-induced hyperhomocysteinemia on endothelium-dependent vasodilation and oxidative status in healthy adults", *Circulation* **101**, 485–490.
[39] Nightingale, A.K., James, P.P., Morris-Thurgood, J., Harrold, F., Tong, R., Jackson, S.K., Cockcroft, J.R. and Frenneaux, M.P. (2001) "Evidence against oxidative stress as mechanism of endothelial dysfunction in methionine loading model", *Am. J. Physiol. Heart Circ. Physiol.* **280**, H1334–H1339.
[40] El Kossi, M.M. and Zakhary, M.M. (2000) "Oxidative stress in the context of acute cerebrovascular stroke", *Stroke* **31**, 1889–1892.
[41] Moselhy, S.S. and Demerdash, S.H. (2003) "Plasma homocysteine and oxidative stress in cardiovascular disease", *Dis. Markers* **19**, 27–31.
[42] Koga, T., Claycombe, K. and Meydani, M. (2002) "Homocysteine increases monocyte and T-cell adhesion to human aortic endothelial cells", *Atherosclerosis* **161**, 365–374.
[43] Li, H., Lewis, A., Brodsky, S., Rieger, R., Iden, C. and Goligorsky, M.S. (2002) "Homocysteine induces 3-hydroxy-3-methylglutaryl coenzyme A reductase in vascular endothelial cells: a mechanism for development of atherosclerosis?", *Circulation* **105**, 1037–1043.
[44] Werstuck, G.H., Lentz, S.R., Dayal, S., Hossain, G.S., Sood, S.K., Shi, Y.Y., Zhou, J., Maeda, N., Krisans, S.K., Malinow, M.R. and Austin, R.C. (2001) "Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways", *J. Clin. Investig.* **107**, 1263–1273.