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ORIGINAL ARTICLE

Free asymmetric dimethylarginine (ADMA) is low in children and adolescents with classical phenylketonuria (PKU)

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Abstract
Introduction Free asymmetric dimethylarginine (ADMA) is a competitive inhibitor of the nitric oxide synthases (NOS). Suppression of nitric oxide (NO) synthesis increases the risk of atherosclerosis. Nevertheless, in the condition of oxidative stress, NOS blockade by ADMA may exert protective effects. Protein metabolism is altered in patients with phenylketonuria (PKU) on dietary treatment and as shown recently, oxidative stress is high in PKU. Since free ADMA concentrations are determined by both protein metabolism and oxidative stress we hypothesized, that free ADMA levels may be elevated in PKU patients.
Design Sixteen patients with PKU on dietary treatment (mean age 10.1±5.2 yrs), and 91 healthy children (mean age 11.6± 3.7 yrs) participated in a cross sectional study.
Results ADMA, total homocysteine (tHcy) and blood glucose were lower and the L-arginine/ADMA ratio was higher in PKU patients compared to controls. No significant correlation

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was present between phenylalanine (Phe) concentrations, protein intake, and lipid profile, history of cardiovascular disease or ADMA.

Discussion In contrast to our hypothesis, ADMA was lower and the L-arginine/ADMA ratio was higher in PKU patients. Therefore, in PKU patients, the regulating function of ADMA on NO synthesis is altered and may thus contribute to oxidative stress.

Abbreviations

ADMA	asymmetric dimethylarginine
CVD	cardiovascular disease
DDAH	dimethylarginine dimethylaminohydrolase
HDL	high density lipoprotein
NOS	nitric oxide synthase(s)
NO	nitric oxide
Phe	phenylalanine
PKU	phenylketonuria
tHcy	total homocysteine

Introduction

Free asymmetric dimethylarginine (ADMA) is a competitive inhibitor of the nitric oxide synthases (NOS) (Beltowski and Kedra 2006). Since nitric oxide (NO) is a potent vasodilatator, protects functional integrity of the endothelium and inhibits platelet aggregation, suppression of NO synthesis is a risk factor for atherosclerosis (Böger et al. 2005). In adults with or at high risk of cardiovascular disease (CVD), ADMA is a predictor of coronary heart disease with an odds ratio of 2.61 for an increase of plasma free ADMA concentrations by 0.1 μmol/L (Schulze et al 2006). Elevated ADMA levels have been measured in patients with diabetes mellitus (Lin 2002), hypercholesterolemia (Engler 2004), chronic renal failure (Kari et al. 1997), hypertension (Goonasekera 2000), and pulmonary hypertension (Gorenflo et al. 2001).

Classical phenylketonuria (PKU, OMIM#261600) is an inborn error of phenylalanine (Phe) metabolism caused by a defect of the hepatic enzyme phenylalanine hydroxylase (EC 1.14.16.1). Phenylalanine hydroxylase catalyses the conversion of the essential amino acid Phe to tyrosine. Enzyme deficiency results in elevated concentrations of Phe. Severe, untreated hyperphenylalaninemia predominantly causes central nervous system dysfunction. Since PKU is included in almost all Newborn Screening programs, dietary treatment is usually initiated soon after birth and recommended lifelong. The PKU diet contains only small amounts of natural, Phe containing protein. To achieve age appropriate protein supply, Phe-free protein substitutes (amino acid mixtures or protein hydrolysates) are added (Burgard et al. 1999). It is known that amino acid absorption and utilization for protein synthesis depend on the mode of ingestion either from intact proteins or from synthetic amino acid preparations (Daenzer et al. 2001).

Since free ADMA is synthesized by dimethylation of protein bound L-arginine residues and consecutive degradation of these proteins, alterations in protein metabolism may have an effect on ADMA concentrations. At the point in time when the study was initiated, we favored the hypothesis that ADMA concentrations would be higher in PKU individuals on dietary treatment. At present, knowledge on ADMA concentrations in PKU is still very limited, but there are arguments both in favor of lower as well as higher ADMA in PKU patients.

The intake of larger proportions of amino acid supplement may increase catabolism (Mönch et al. 1996) which argues in favor of higher ADMA. Nevertheless, lower ADMA concentrations have been shown in a recent study in 52 individuals with PKU compared to age and sex matched healthy controls, but details on daily protein intake are not provided (Kanzelmeyer et al. 2011). Additionally there is increasing evidence that oxidative stress markers are elevated and antioxidant activity is lower in PKU patients (Sanayama et al. 2011). Oxidative stress increases the activity of arginine methylating and may thus increase ADMA concentrations (Sydow and Münzel 2003). In contrast, Sanayama et al. most recently found lower ADMA levels in PKU patients and suggest the reverse: low ADMA perpetuates oxidative stress (Sanayama et al. 2011).

Design and methods

Cross sectional study including children and adolescents with PKU and healthy controls between 2 and 18 yrs of age. The study protocol had been approved by the local ethics committee (protocol number 2006-3/1). Informed, written consent / assent were obtained from all participants >8 years and their parents / guardians. Blood sampling was done as part of routine visits.

All PKU patients were recruited from the Metabolic Outpatient's Clinic of the University Children's Hospital, Vienna Medical School. For the control group, healthy children and adolescents scheduled for elective ENT, general surgery or allergies testing respectively were recruited.

In all participants, body weight, length, body mass index (BMI), blood pressure, creatinine, blood glucose, ADMA, total homocysteine (tHcy), folate, L-arginine, Phe, family and individual history of CVD were investigated. Additionally, dietary intake of natural protein and L-amino acids (g/kg/d) were assessed in PKU patients.

Body weight was measured by electronic scales with 0.05 kg degree of accuracy. Height was measured using a stadiometer with 0.5 cm degree of accuracy. The BMI was

calculated using the equation BMI=weight (kg) / height (m) ^{2}. Age, gender, and family and individual history of CVD were recorded using a standardized data sheet. Blood pressure was measured according to the Riva-Rocci method. The PKU patients and/or their parents recorded dietary intake for 3 days, including one holiday or Sunday respectively and two working days. Total, natural protein and L-amino acid intake were calculated by an experienced dietician.

Blood samples (5 ml) were taken after an overnight fast. Arginine and Phe plasma levels were analyzed by ion exchange chromatography (Biotronik LC3000). In addition arginine and phe levels were analyzed in dried blood spots for clinical management using stable isotope LC-MS/MS (data not shown). ADMA plasma levels in all samples were finally analyzed by an established ELISA method (DLD, Hamburg) (Schulze et al. 2005) following the initial use of a stable isotope dilution LC-MS/MS technique. The latter method was shown not to be sufficiently sensitive (data not shown).

Plasma tHcy concentrations were determined using an automated fluorescence polarization immunoassay (FPIA, Abbott IMx® analyzer) while folate concentrations were measured with a Microparticle Enzyme Immunoassay (Abbott Imx® Analyzer, Abbott Laboratories, Abbott Park, Illinois, USA). Serum creatinine, triglycerides, cholesterol and high density lipoprotein (HDL) concentrations as well as blood glucose were measured at 37°C using standard laboratory assays.

ADMA concentrations, as the primary study parameter were compared in a confirmative way between PKU patients versus healthy controls applying a two-sided t-test at a significance level of 0.05. All other characteristics of PKU patients and healthy controls were compared using either t-test, Mann-Whitney U test, chi-square or Fisher's exact test as appropriate. Associations of ADMA with other parameters were assessed by calculating Spearman correlation coefficients, separately for PKU patients and healthy controls.

Results

A total of 107 children and adolescents, 16 patients with PKU (mean age 10.1±5.2 yrs, 7 females) and 91 controls (mean age 11.6±3.7 yrs, 32 females) participated in the study.

Diastolic blood pressure, tHcy, ADMA (all: $p<0.0001$), and blood glucose ($p=0.008$) were higher in healthy controls while L-arginine/ADMA ratio ($p=0.001$), triglycerides ($p=0.008$), and Phe ($p<0.0001$) were higher in PKU patients. No significant between-group differences were identified for age, BMI, systolic blood pressure, creatinine, cholesterol, HDL, folic acid and arginine (Table 1).

Correlation analysis revealed significant correlations between ADMA and blood glucose in controls ($p=0.002$). Only in the PKU patients, tHcy had a significant negative correlation with ADMA ($p=0.029$). No significant correlation was

Table 1 Medians and interquartile ranges for skewed, and means and standard deviation (SD) for normally distributed variables in patients with PKU and controls [n.s. = not significant]

Parameter	Unit	PKU (N=16; 7 females)		Controls (N=91; 32 females)		p-value
		Mean	SD	Mean	SD	
Age	years	10.1	5.2	11.6	3.7	n.s.
BMI	kg/m2	19.5	4.5	19.2	4.3	n.s.
Protein intake	g/kg/d	1.3	0.4	–	–	–
Natural protein intake	g/kg/d	0.23	0.11	–	–	–
L-amino acid intake	g/kg/d	1.1	0.25	–	–	–
RR systolic	mmHg	108	18	117	15	n.s.
RR diastolic	mmHg	64	12	76	11	p<0.0001
Cholesterol	mmol/L	3.57	0.83	3.7	0.7	n.s.
HDL	mmol/L	1.22	0.28	1.37	0.36	n.s.
Glucose	mmol/L	4.44	0.44	4.88	0.72	p=0.008
		Median	25-75. Quartile	Median	25-75. Quartile	
ADMA	µmol/L	0.43	0.36-0,5	0.64	0.55-0.76	p<0.0001
L-arginine	µmol/L	56	47.5-91	59	37-80	n.s.
L-arginine/ADMA		155	98-213	86	57-125	p=0.001
Phe	µmol/L	809	400-919	62	50-80	p<0.0001
Triglycerides	mmol/L	0.98	0.75-1.42	0.69	0.5-0.98	p=0.008
tHcy	µmol/L	6.1	3.6-9.8	12.4	8.3-17.5	p<0.0001
Folic acid	nmol/L	5.8	4.5-10.6	6	4.4-7.9	n.s.
Creatinine	µmol/L	52	39-61	53	35-53	n.s.

present between lipid profile or a history of cardiovascular death/disease and ADMA.

Discussion

Newborn screening for PKU was initiated in Austria in 1966 and the first patients who were detected and treated with a PKU diet have now reached middle age. Dietary treatment meant a revolution considering the prognosis of PKU, and compliant patients have a normal intellectual and social development (Channon et al. 2007). Nevertheless, there are concerns about the continuous intake of artificial protein sources and especially since new treatment approaches evolve, the long term safety of dietary treatment and its potential impact on the classical diseases of middle age (e.g., CVD) are to be evaluated. Consequently we studied free ADMA -a risk factor for CVD- in treated PKU patients and healthy controls under the hypothesis that ADMA might be higher in PKU patients due to the altered protein metabolism induced by their diet containing large amounts of synthetic amino acids (Daenzer et al. 2001).

In contrast to our initial hypothesis ADMA was significantly lower in PKU patients. THcy concentrations were low in the PKU group, so there is no evidence for a reduced methyl-group supply due to an inhibition of the methionine-Hcy circle which may have resulted in impaired ADMA generation (Beltowski and Kedra 2006). Nevertheless, S-adenosylmethionine or S-adenosylhomocysteine has not been measured in this study. It has been shown in an experimental setting in patients with diabetes type 1, that both ADMA and arginine concentrations were decreased in coincidence with impaired protein catabolism (Marcovecchio et al. 2008). Our data do not completely fit into this pattern since in our PKU population only ADMA was decreased.

In the PKU group, blood glucose concentrations as well as ADMA were lower compared to controls and this may reflect the mechanism that insulin preserves the activity of the ADMA degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Anderssohn 2010), a finding supported by observations in a rabbit model of critical illness (Ellger 2008).

In an earlier study in 52 PKU patients, both lower ADMA and slightly lower arginine levels were observed. The authors attribute the lower ADMA concentrations to the lifelong restriction of natural protein; nevertheless details on the diet in their patients are not provided (Kanzelmeyer 2011). We found no correlation of ADMA with the daily portion of natural protein intake or the amount of amino acid supplements; therefore our data do not support this argument. In our study, arginine levels were not significantly different from those in controls. Therefore the L-arginine/ADMA ratio -reflecting the relation of NO substrate to inhibitor - was significantly higher in PKU patients. This contradicts the conclusion that NO metabolism is not altered in PKU patients (Kanzelmeyer et al. 2011).

Lücke et al. 2007 measured ADMA levels of ~1.0 μmol/L in young healthy children. In their cohort ADMA correlates inversely with age (Lücke et al. 2007). In contrast, in adults a rise of ADMA concentrations by 0.1 μmol/L results in an odds ratio of 2.61 for coronary heart disease with mean ADMA levels in cases of 0.7 μmol/L (Schulze et al. 2006). Following the concept, that higher ADMA levels are a physiological phenomenon in children but in contrast are associated with disease in adults, the question arises, whether high free ADMA might under certain circumstances exert positive effects. In the presence of oxidative stress, NOS becomes "uncoupled" and thus shifts from the production of NO towards the generation of free radicals such as superoxide that in turn may react with NO to produce peroxynitrite. Therefore, NO is not only a vasoprotective agent but under different circumstances a player in the initiation and sustainment of oxidative stress. In this case, inhibition of NOS by higher ADMA levels may protect the system from the perpetuation of oxidative stress (Pitocco et al. 2009, Anderssohn et al. 2010). Recently a significant elevation of oxidative stress markers and decrease of antioxidant activity in the presence of low ADMA levels has been observed in adolescents and adults with PKU and the authors conclude that low ADMA, resulting in impaired regulation of NO production, may fuel oxidative stress (Sanayama et al. 2011).

In conclusion, our data support the finding that ADMA levels are significantly lower in PKU patients (Kanzelmeyer 2011). At present, on the basis of data derived from small samples, this finding is not completely understood. Facilitated ADMA degradation in the context of low blood glucose may be of significance. The kinetics of ADMA differs between adults and children and higher ADMA might be advantageous for young children. The relation between ADMA and oxidative stress in PKU patients requires further investigation.

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