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ORIGINAL ARTICLE

The L-arginine/NO pathway, homoarginine, and nitrite-dependent renal carbonic anhydrase activity in young people with type 1 diabetes mellitus

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Abstract High circulating levels of asymmetric dimethylarginine (ADMA) and low circulating levels of homoarginine (hArg) are known cardiovascular risk factors in adults. While in adults with type 1 diabetes mellitus (T1DM) circulating ADMA is significantly elevated, in children and adolescents the reported ADMA data are contradictory. In 102 children with T1DM and 95 healthy controls (HC) serving as controls, we investigated the L-arginine (Arg)/nitric oxide (NO) pathway. Children with T1DM were divided into two groups, i.e., in children with newly diagnosed diabetes mellitus [T1DM-ND; $n = 10$; age, 8.8 (4.4–11.2) years; HbA_{1c}, 13 (8.9–13.9) %] and in those with long-term treatment [T1DM-T; $n = 92$; age, 12.5 (10.5–15.4) years; HbA_{1c}, 8.0 (7.2–8.6) %]. The age of the HC was 11.3 (8–13.3) years. Amino acids and NO metabolites of the Arg/NO pathway, creatinine and the oxidative stress biomarker malondialdehyde (MDA) were measured by GC–MS or GC–MS/MS. Plasma hArg, ADMA and the hArg/ADMA molar ratio did not differ between the T1DM and HC groups. There was a significant difference between T1DM-T and HC with regard to plasma nitrite [0.53 (0.48–0.61) vs 2.05 (0.86–2.36) μM, $P < 0.0001$] as well as to urinary nitrite [0.09 (0.06–0.17) vs 0.22 (0.13–0.37) μmol/mmol creatinine, $P < 0.0001$]. Plasma, but not urinary nitrite, differed between T1DM-ND and HC [0.55 (0.50–0.66) vs 2.05 (0.86–2.36) μM, $P < 0.0001$]. Plasma MDA did not differ between the groups. The urinary nitrate-to-nitrite molar ratio ($U_{NOX}R$), a measure of nitrite-dependent renal carbonic anhydrase (CA) activity, was higher in T1DM-T [1173 (738–1481), $P < 0.0001$] and T1DM-ND [1341 (1117–1615), $P = 0.0007$] compared to HC [540 (324–962)], but did not differ between T1DM-T and T1DM-ND ($P = 0.272$). The lower nitrite excretion in the children with T1DM may indicate enhanced renal CA-dependent nitrite reabsorption compared with healthy children. Yet, lower plasma nitrite concentration in the T1DM patients may have also contributed to the higher $U_{NOX}R$. Patients' age correlated positively with plasma hArg and hArg/ADMA and urinary DMA/ADMA. Plasma ADMA and urinary ADMA, DMA, nitrite and nitrate correlated negatively with age of the T1DM-T children. Significant correlations were found between plasma hArg and plasma Arg ($r = 0.468$, $P < 0.0001$), and urinary DMA ($r = -0.426$, $P = 0.0001$), ADMA ($r = -0.266$, $P = 0.021$) and nitrate ($r = -0.234$, $P = 0.043$). Plasma hArg correlated positively with age at diagnosis ($r = +0.337$, $P = 0.002$). ADMA, but not hArg, correlated with HbA_{1c} in T1DM-T ($r = -0.418$, $P < 0.0001$) and T1DM-ND ($r = +0.879$, $P = 0.0016$). The greatest differences between T1DM-T and T1DM-ND were observed for urinary ADMA, DMA/ADMA ratio, nitrite and nitrate. The Arg/NO pathway is altered in T1DM in childhood and adolescence, yet the role and the importance of hArg and ADMA in T1DM remain to be elucidated. In young

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T1DM patients, oxidative stress (lipid peroxidation) is not elevated.

Keywords Asymmetric dimethylarginine · Blood · Carbonic anhydrase · Diabetes · Homoarginine · Kidney · Nitrite · Urine

Abbreviations

ADMA	Asymmetric dimethylarginine
Arg	L-Arginine
BMI	Body mass index
CA	Carbonic anhydrase
DDAH	Dimethylarginine dimethylaminohydrolase
DMA	Dimethylamine
GFR	Glomerular filtration rate
hArg	L-Homoarginine
HC	Healthy controls
MDA	Malondialdehyde
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial NOS
iNOS	Inducible NOS
nNOS	Neuronal NOS
$P_{NOx}R$	Plasma nitrate-to-nitrite molar ratio
QC	Quality control
T1DM	Type 1 diabetes mellitus
T1DM-ND	Newly diagnosed T1DM
T1DM-T	Treated T1DM
$U_{NOx}R$	Urinary nitrate-to-nitrite molar ratio

Introduction

Nitric oxide (NO) is a free soluble gaseous molecule, originally known as the endothelium-derived relaxing factor (Palmer et al. 1987). Three main types of the NO synthase (NOS) enzyme are known, the constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS) as well as the inducible NOS (iNOS). These enzymes oxidize the imino group of the terminal guanidine group of L-arginine (Arg) to NO and L-citrulline (Marletta 1993). NO is a potent vasodilator, regulates blood pressure, inhibits platelet aggregation, and is involved in neurotransmission (Moncada and Higgs 2006). In vivo, NO is rapidly oxidized to nitrate and nitrite which circulate in blood and are excreted in urine. Circulating and urinary nitrate and nitrite are commonly used as measures of NO synthesis (Tsikas 2000, 2015). Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NOS. ADMA is produced from the methylation of Arg by the enzyme *N*-protein arginine-methyltransferase (Tran et al. 2003). Arg and its homolog homoarginine (hArg) are substrates of NOS. ADMA is hydrolyzed to dimethylamine (DMA) by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (Vallance and Leiper 2004). High circulating levels of ADMA and low circulating levels of homoarginine (hArg) are known cardiovascular risk factors in adults (Böger 2006; Böger et al. 2009; Kielstein et al. 2004; März et al. 2010). High ADMA levels have been found in adults with type 1 diabetes mellitus (T1DM) compared to healthy controls (Altinova et al. 2007; Marcovecchio et al. 2011). In previous studies, we reported on developmental changes in the Arg/NO pathway from infancy to adulthood, showing a decrease in plasma ADMA levels with age (Lücke et al. 2007), as well as in metabolic diseases (Lücke et al. 2006, 2008; Chobanyan-Jürgens et al. 2012a; Kanzelmeyer et al. 2012) (Fig. 1).

Concerning circulating levels of ADMA in children and adolescents with T1DM, there are contradictory data (Heilman et al. 2009; Głowińska-Olszewska et al. 2010; Huemer et al. 2011; Jehlicka et al. 2009). Other metabolites of the Arg/NO family have not been studied by these groups. In young adults, infusion of insulin caused a significant decrease in circulating ADMA levels (Marcovecchio et al. 2008). Such data for children and adolescents are lacking. In previous studies, we found that renal carbonic anhydrase (CA) is involved in the reabsorption of nitrite (Chobanyan-Jürgens et al. 2012b) and that this nitrite-dependent CA activity can be inhibited by endogenous and exogenous factors including drugs such as acetazolamide and *N*-acetyl-L-cysteine (Tsikas et al. 2014). This may be of particular importance because nitrite is considered to be an NO reservoir, and proteins such as hemoglobin, xanthine oxidoreductase and CA may convert nitrite to NO (Aamand et al. 2009).

In the present study, we investigated the status of the Arg/NO pathway by measuring several representative biochemical parameters including hArg in plasma and urine of children and adolescents with T1DM in comparison to healthy non-diabetic children.

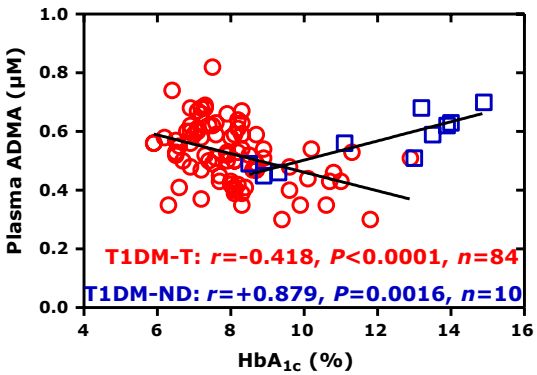

Fig. 1 Spearman correlation between plasma ADMA concentration and HbA_{1c} in the patients with T1DM-T and T1DM-ND in the present study. Inserted lines were obtained by linear regression analysis. T1DM-T: $y = 0.779 - 0.032x$, $r^2 = 0.178$, P; $y = 0.177 + 0.033x$, $r^2 = 0.745$

Materials and methods

Subjects

In the present study, we investigated the Arg/NO pathway in 102 children (57 boys, 45 girls) with T1DM and in 95 healthy control children. The study was designed as a prospective cross-sectional study and was approved by the Ethics Committee of the University of Bochum. The study was performed in accordance with the guidelines of the Declaration of Helsinki and of Good Clinical Practice. Written and informed consent was given by parents and children. Children and adolescents up to 20 years of age with T1DM were included. Children with newly diagnosed T1DM (T1DM-ND; first manifestation) were analyzed separately from those under long-term insulin treatment (T1DM-T). The exclusion criteria from the study were acute or chronic liver, kidney or cardiac diseases, malignoma, arterial hypertension, dystrophy, pregnancy, intake of medications, or consumption of seafood within the preceding 24 h of testing to avoid nutritional contribution to DMA levels (Tsikas et al. 2007). The clinical data of the investigated children are shown in Table 1. The age of the HC was [median (25th–75th percentile)] 11.3 (8–13.3) years. All subjects of the HC group did not have hypertension, hypercholesterolemia, hypertriglyceridemia or excess weight, and had a normal renal function.

Table 1 Clinical data of children with T1DM enrolled in the study

Parameter	T1DM-T	T1DM-ND
Number of children (*n*)	92	10
Age (years)	12.5 [10.5–15.4]	8.8 [4.4–11.2]
Age at diagnosis (years)	7.13 [4.0–9.8]	6.3 [3.5–9.8]
Duration of diabetes (years)	4.99 [2.25–8.2]	Not applicable
Systolic blood pressure (mmHg)	113 [106–119]	108 [102–124]
Diastolic blood pressure (mmHg)	65 [59–71]	65.5 [63–74]
BMI (cm/kg^2)	20.1 [17.8–22.8]	14.7 [13.7–20.2]
HbA_{1c} (%)	8.0 [7.2–8.6]	13.0 [8.9–13.9]
Hb (g/dL)	13.4 [12.9–14.1]	13.3 [12.2–14.0]
HDL cholesterol (mg/dL)	58 [51–66]	71 [58–77]
LDL cholesterol (mg/dL)	89 [77–108]	115 [87–135]
Triglycerides (mg/dL)	98.5 [72–145]	75 [67–105]
Cholesterol (mg/dL)	170 [147–192]	168 [95–226]
Creatinine (mg/dL)	0.65 [0.56–0.78]	0.53 [0.41–0.73]
Urine albumin (mg/dL)	0.29 [0.0–0.6]	0.12 [0.0–0.6]
Creatinine clearance (mL/min)	118 [101–153]	115 [34–138]

Data are given as median [25th–75th percentile]

Biochemical analyses

Venous blood and urine samples were taken during annual clinical examination of the diabetic patients. After acquisition, the samples were placed on ice until immediate blood centrifugation. Plasma and urine samples were stored frozen at −80 °C until analysis. Urinary excretion of all biochemical parameters was corrected for creatinine excretion. Glomerular filtration rate (GFR) was calculated by using the Cockcroft–Gault formula. ADMA in plasma and urine was measured by GC–MS/MS (Tsikas et al. 2003). Urinary DMA (Tsikas et al. 2007) and creatinine (Tsikas et al. 2010a, b) were measured by GC–MS. Arg in plasma was determined by GC–MS as described previously (Tsikas et al. 2003). Nitrite and nitrate in plasma and urine were measured by GC–MS as described elsewhere (Tsikas 2000). hArg in plasma samples was determined by GC–MS/MS (Kayacelebi et al. 2014). MDA was determined in plasma by GC–MS/MS as described elsewhere (Dreissigacker et al. 2010). Quality control (QC) samples were analyzed alongside study samples. In the QC samples, all biochemical parameters were determined with accuracy (bias, %) and imprecision (relative standard deviation, %) of less than ±20 % and ≤20 %, respectively. The QC data indicate that the analytical results in the plasma and urine samples of the study are valid.

Statistical analysis

The D'Agostino and Pearson omnibus K2 and Kolmogorov–Smirnov normality tests were used to evaluate data distribution. The non-parametric Mann–Whitney test was used for statistical analysis of variables which were not normally distributed. For the sake of simplicity and because the majority of the parameters were not normally distributed, all data were presented as median (25th–75th percentile). Spearman's correlation coefficient for non-normally distributed variables was assessed. Two-tailed P values <0.05 were considered as statistically significant. Pairwise comparisons (T1DM-T vs HC, T1DM-ND vs HC, and T1DM-T vs T1DM-ND) were made with Bonferroni correction. For each pairwise comparison, $P < 0.017$ was considered as statistically significant ($P = 0.05$ divided by 3, i.e., the number of comparisons). All calculations were performed using GraphPad Prism software (GraphPad Prism Software Inc. San Diego, CA, USA).

Results

Table 1 summarizes the clinical data of the children with T1DM investigated in the present study. One group of the children with T1DM was under long-term insulin treatment

(T1DM-T). The second T1DM group consisted of children with newly diagnosed diabetes (T1DM-ND). Only two diabetic patients of the T1DM-T group had microalbuminuria which serves as an early marker of diabetic nephropathy (Al-Agha et al. 2013). The concentration of the circulating and urinary biochemical parameters of the Arg/NO pathway measured in our children with T1DM and healthy non-diabetic controls and the results of the statistical analyses are summarized in Table 2 (T1DM-T vs HC), Table 3 (T1DM-ND vs HC) and Table 4 (T1DM-T vs T1DM-ND). The results from correlation analyses between the age of the children or plasma hArg concentration and various biochemical and clinical parameters are summarized in Tables 5 and 6.

The plasma Arg concentration differed between T1DM-T and HC [89.6 (74–105) vs 76.1 (63.1–85.4) µM, $P < 0.0001$] (Table 2). Plasma levels of ADMA and hArg and the hArg/ADMA ratio did not differ among the T1DM-T and T1DM-ND groups. Also, there were no statistical differences between T1DM-T and HC groups or between T1DM-ND and HC groups with respect to plasma ADMA and hArg. A statistically significant difference ($P = 0.004$) was observed between T1DM-T and HC for urinary DMA: 30.5 (24.5–37.5) µmol/mmol creatinine vs 41.1 (24.7–57.5) µmol/mmol creatinine, but not for the urinary DMA/ADMA ratio (Table 2).

Table 2 Circulating and urinary biochemical parameters in the T1DM-T and HC groups

Matrix/biomarker	T1DM-T	HC	*P* value
Plasma (µM or nM)			
L-Arginine	89.6 [74–105]	76.1 [63.1–85.4]	**<0.0001**
ADMA	0.52 [0.45–0.60]	0.57 [0.50–0.64]	0.065
hArg	1.57 [1.20–1.90]	1.67 [1.41–1.94]	0.243
hArg/ADMA	2.94 [2.17–3.85]	3.13 [2.38–3.85]	0.820
Nitrite	0.53 [0.48–0.61]	2.05 [0.86–2.36]	**<0.0001**
Nitrate	35.1 [31.0–44.4]	33.7 [27.0–42.3]	0.667
MDA (nM)	110 [83–161]	119.0 [72.5–161.5]	0.759
Urine (µmol/mmol creatinine)			
ADMA	5.32 [4.23–6.78]	6.57 [5.15–8.87]	**0.0007**
DMA	30.5 [24.5–37.5]	41.1 [24.7–57.5]	**0.004**
DMA/ADMA	5.84 [4.72–7.01]	6.12 [4.27–9.64]	0.700
Nitrite	0.09 [0.06–0.17]	0.22 [0.13–0.37]	**<0.0001**
Nitrate	101 [72–158]	117 [85–164]	0.115
Urinary nitrate/nitrite ratio ($U_{NOx}R$)	1173 [738–1481]	540 [324–962]	**<0.0001**
Plasma nitrate/nitrite ratio ($P_{NOx}R$)	68.9 [55.4–84.2]	20.4 [14–41.2]	**<0.0001**
$U_{NOx}R/P_{NOx}R$	17.0	26.5	

Data are given as median [25th–75th percentile]

Statistically significant *p* values are in bold

Table 3 Circulating and urinary biochemical parameters in the T1DM-ND and HC groups

Matrix/biomarker	T1DM-ND	HC	*P* value
Plasma (µM or nM)			
L-Arginine	80.4 [74.2–101]	76.1 [63.1–85.4]	0.076
ADMA	0.59 [0.49–0.68]	0.57 [0.50–0.64]	0.699
hArg	1.68 [1.26–1.77]	1.67 [1.41–1.94]	0.572
hArg/ADMA	2.70 [2.50–3.13]	3.13 [2.38–3.85]	0.362
Nitrite	0.55 [0.50–0.66]	2.05 [0.86–2.36]	**<0.0001**
Nitrate	35.3 [30.7–50.5]	33.7 [27.0–42.3]	0.473
MDA (nM)	120 [103–193]	119.0 [72.5–161.5]	0.611
Urine (µmol/mmol creatinine)			
ADMA	10.2 [5.64–13.0]	6.57 [5.15–8.87]	0.083
DMA	40.3 [28.1–46.3]	41.1 [24.7–57.5]	0.661
DMA/ADMA	4.48 [3.64–5.55]	6.12 [4.27–9.64]	0.054
Nitrite	0.18 [0.11–0.33]	0.22 [0.13–0.37]	0.585
Nitrate	176 [149–390]	117 [85–164]	0.017
Urinary ntrate/nitrite ratio ($U_{NOx}R$)	1341 [1117–1615]	540 [324–962]	**0.0007**
Plasma nitrate/nitrite ratio ($P_{NOx}R$)	67.8 [65.0–84.2]	20.4 [14–41.2]	**<0.0001**
$U_{NOx}R/P_{NOx}R$	19.8	26.5	

Data are given as median [25th–75th percentile]

Statistically significant *p* values are in bold

Table 4 Circulating and urinary biochemical parameters in the T1DM-T and T1DM-ND groups

Matrix/biomarker	T1DM-T	T1DM-ND	*P* value
Plasma (µM)			
L-Arginine	89.6 [74–105]	80.4 [74.2–101]	0.770
ADMA	0.52 [0.45–0.60]	0.59 [0.49–0.68]	0.085
hArg	1.57 [1.20–1.90]	1.68 [1.26–1.77]	0.796
hArg/ADMA	2.94 [2.17–3.85]	2.70 [2.50–3.13]	0.482
Nitrite	0.53 [0.48–0.61]	0.55 [0.50–0.66]	0.573
Nitrate	35.1 [31.0–44.4]	35.3 [30.7–50.5]	0.734
MDA (nM)	110 [83–161]	120 [103–193]	0.234
Urine (µmol/mmol creatinine)			
ADMA	5.32 [4.23–6.78]	10.2 [5.64–13.0]	**0.003**
DMA	30.5 [24.5–37.5]	40.3 [28.1–46.3]	0.100
DMA/ADMA ratio	5.84 [4.72–7.01]	4.48 [3.64–5.55]	**0.013**
Nitrite	0.09 [0.06–0.17]	0.18 [0.11–0.33]	**0.029**
Nitrate	101 [72–158]	176 [149–390]	**0.006**
Urinary nitrate/nitrite ratio ($U_{NOx}R$)	1173 [738–1481]	1341 [1117–1615]	0.272
Plasma nitrate/nitrite ratio ($P_{NOx}R$)	68.9 [55.4–84.2]	67.8 [65.0–84.2]	0.517
$U_{NOx}R/P_{NOx}R$	17.0	19.8	

Data are given as median [25th–75th percentile]

Statistically significant *p* values are in bold

Table 5 Summary of Spearman correlations between age of the T1DM group and HC and some biochemical parameters

Parameter	T1DM-T	*P*	*n*	T1DM-ND	*P*	*n*	HC	*P*	*n*
	r			*r*			*r*		
P-ADMA	−0.548	<0.0001	86	−0.770	0.013	10	−0.211	0.046	90
U-ADMA	−0.722	<0.0001	77	−0.767	0.021	9	−0.281	0.015	75
U-DMA	−0.370	0.0009	77	−0.667	0.083	8	+0.113	0.384	61
U-DMA/ADMA	+0.423	0.0001	78	+0.583	0.108	9	+0.355	0.004	64
P-hArg	+0.409	<0.0001	86	+0.006	1.000	10	+0.101	0.624	26
P-hArg/ADMA	+0.641	<0.0001	86	+0.455	0.192	10	+0.163	0.435	25
U-Nitrite	−0.473	<0.0001	77	−0.383	0.313	9	−0.239	0.045	71
U-Nitrate	−0.553	<0.0001	77	−0.500	0.178	9	−0.294	0.010	75

P plasma, *U* urine

Table 6 Summary of significant Spearman correlations found between plasma hArg and other parameters in the T1DM-T group

Parameter	*r*	*P*	*n*
P-Arg	+0.468	<0.0001	87
U-DMA	−0.426	0.0001	75
U-ADMA	−0.266	0.021	75
U-Nitrate	−0.234	0.043	75
U-Nitrite	−0.213	0.067	75
Hemoglobin	+0.199	0.071	83
Triglycerides	−0.225	0.043	81
Age at diagnosis	+0.337	0.002	81
Body mass index	+0.243	0.026	84
Creatinine clearance	+0.243	0.036	75

P plasma, *U* urine

There were remarkable statistically significant differences between T1DM-T and HC for nitrite both in plasma [0.53 (0.48–0.61) vs 2.05 (0.86–2.36) μM, $P < 0.0001$] (Table 2) and urine [0.09 (0.06–0.17) vs 0.22 (0.13–0.37) μmol/mmol creatinine, $P < 0.0001$] (Table 2). We also found a significantly higher urinary nitrate-to-nitrite molar ratio ($U_{NOx}R$) in the T1DM-T and T1DM-ND groups compared to the HC group: 1173 (738–1481) and 1341 (1117–1615) vs 540 (324–962) (Tables 2, 3, 4). $U_{NOx}R$ did not differ between the T1DM-ND and T1DM-T groups ($P = 0.272$). This also applies to the plasma nitrate-to-nitrite molar ratio ($P_{NOx}R$) in the T1DM-T and T1DM-ND groups compared to the HC group: 68.9 (55.4–84.2) and 67.8 (65.0–84.2) vs 20.4 (14.0–41.2) (Tables 2, 3, 4). The mean ratio of $U_{NOx}R$ to $P_{NOx}R$ ($U_{NOx}R/P_{NOx}R$) was 17 in T1DM-T, 19.8 in T1DM-ND and 26.5 in HC.

The T1DM-T, T1DM-ND and HC groups behaved differently with respect to correlation between age of the children and the biochemical parameters of the Arg/NO pathway in plasma and urine (Table 5). Considerable correlations were observed in the T1DM-T group. Thus, plasma hArg concentration, and plasma hArg/ADMA and urinary DMA/ADMA ratios correlated positively with age at diagnosis of the T1DM-T children. Considerable, yet negative, correlations were observed between the age of the T1DM-T children and plasma ADMA or urinary excretion of ADMA, DMA, nitrite and nitrate. These correlations were less strong with the age of the HC. In the T1DM-ND group, considerable negative correlations were found between the age of diagnosis and plasma and urinary ADMA concentration, despite the considerably smaller number of children in the T1DM-ND compared to the T1DM-T group (Table 5).

Given the pronounced correlations in the T1DM-T group, we tested for correlations of biochemical and clinical parameters with the plasma hArg concentration in this group (Table 6). Moderate, albeit opposite correlations were observed between plasma hArg and Arg ($r = 0.468$, $P < 0.0001$) or urinary DMA ($r = -0.426$, $P = 0.0001$). Plasma ADMA correlated with plasma Arg ($r = 0.455$, $P < 0.0001$). Weaker correlations were observed between plasma hArg and urinary ADMA, nitrate and nitrite. Plasma hArg and triglycerides correlated weakly negatively. In contrast, plasma hArg correlated positively with the age at diagnosis, body mass index (BMI), creatinine clearance or hemoglobin ($r = 0.199$, $P = 0.071$). $U_{NOx}R$ correlated with $P_{NOx}R$ in the T1DM-T group ($r = 0.265$, $P = 0.022$), but not in the HC group ($r = 0.222$, $P = 0.175$).

Spearman´s correlation analysis between plasma ADMA concentration and HbA_{1c} indicates a negative correlation in the T1DM-T group ($r = -0.418$, $P < 0.0001$), but a positive and stronger correlation in the patients with T1DM-ND ($r = +0.879$, $P < 0.0016$).

The plasma MDA concentration did not differ between the groups indicating no elevated oxidative stress and notable lipid peroxidation, in T1DM in childhood/adolescence, and was close to that measured in healthy adults (Modun et al. 2012).

Discussion

General issues of the L-Arg/NO pathway and hArg

hArg and ADMA are cardiovascular risk factors in adults (Vallance 2001; Böger 2004, 2006; Siroen et al. 2006; März et al. 2010; Pilz et al. 2015). ADMA is significantly elevated in adults with T1DM with or without diabetic nephropathy (Altinova et al. 2007; Cighetti et al. 2009; Tarnow et al. 2004; Vallance et al. 1992). To our knowledge, no data are available in the literature for hArg in diabetic adults. Reports on circulating ADMA and hArg concentrations from studies dealing with children and adolescents with T1DM are in part contradictory (Altinova et al. 2007; Huemer et al. 2011; Jehlicka et al. 2009; Głowińska-Olszewska et al. 2010; Krebs et al. 2015). In the present study, we investigated the status of the Arg/NO pathway in children and adolescents with T1DM in comparison to non-diabetic HC. The T1DM group consisted of two groups, i.e., a small group ($n = 10$) of newly diagnosed T1DM (i.e., T1DM-ND) and a larger group ($n = 92$) of children with T1DM under long-term insulin therapy (T1DM-T). The status of the Arg/NO pathway was determined quantitatively by measuring in plasma and urine a series of biochemical parameters as measures of NO biosynthesis and availability. These parameters include the amino acid Arg, its metabolites hArg and ADMA, DMA, the metabolite formed from the DDAH-catalyzed hydrolysis of ADMA and the NO metabolites nitrite and nitrate. Finally, MDA was determined in plasma as a biomarker of oxidative stress, specifically lipid peroxidation.

The main findings of the present study are that the amino acids Arg, hArg and ADMA are not significantly different in our T1DM-T and T1DM-ND children and adolescents compared to non-diabetic HC. The differences regarding plasma and urinary nitrite and nitrate are more pronounced. These issues are discussed below in detail.

Amino acids of the L-Arg/NO pathway

Several groups have measured ADMA, hArg and HbA_{1c} and tested for potential correlation between circulating ADMA or hArg and HbA_{1c} in children suffering from T1DM (Heilman et al. 2009; Głowińska-Olszewska et al. 2010; Huemer et al. 2011; Marcovecchio et al. 2011; Önder et al. 2014; Krebs et al. 2015) (Table 7). In a recently reported study on 28 children with T1DM (median age, 14.5 years) and 41 healthy controls (median age, 14 years), median circulating hArg (0.9 vs 1.42 µM, difference, 37 %) and ADMA (0.4 vs 1.06 µM; difference, 62 %) were found to be considerably lower ($P < 0.001$ each) in T1DM compared to healthy non-diabetic children (Krebs et al. 2015). To our knowledge, this is the first study to report on hArg concentrations in children with T1DM. The ADMA and hArg concentrations reported by this group disagree with our observations from the present study with regard to ADMA and hArg and with regard to ADMA in previous studies. Thus, the average ADMA plasma concentration of 1.06 µM in healthy controls (Krebs et al. 2015) is almost two times higher than that measured in the HC group of the present study and in healthy children and adolescents of a previous study (Lücke et al. 2007). Furthermore, both the physiological variability and the extent of effects of disease or pharmacological treatment on circulating ADMA (Horowitz and Heresztyn 2007; Tsikas 2008) and hArg (Tsikas et al. 2014; Pilz et al. 2015) concentrations are very low. In the present study, the difference between the groups was of the order of 13 % for plasma ADMA and only 7 % for hArg. Analytical issues rather than differences in studied populations are likely to have contributed to the discrepancies mentioned above. The methods of analysis of ADMA have been reviewed and discussed (see Horowitz and Heresztyn 2007; Tsikas 2008; Martens-Lobenhoffer and Bode-Böger 2012). Some studies which reported circulating ADMA concentrations comparable to those measured by us using GC–MS/MS led to the conclusion that

Table 7 Correlation ADMA - HbA1c and hArg - HbA1c : Results of different studies

ADMA (µM)		Correlation with HbA_{1c}	hArg (µM)		Control correlation with HbA_{1c}	Reference
T1DM	Control		T1DM	Control		
0.55	0.67	None	n.r.		n.r.	Heilman et al. (2009)
0.69	0.70	None	n.r.		n.r.	Głowińska-Olszewska et al. (2010)
0.48	0.64	Negative	n.r.		n.r.	Huemer et al. (2011)
0.47	0.46	Negative	n.r.		n.r.	Marcovecchio et al. (2011)
0.51	0.57	Positive	n.r.		n.r.	Önder et al. (2014)
0.40	1.06	Negative	0.90	1.42	Negative	Krebs et al. (2015)
0.52	0.57	Negative	1.57	1.67	None	Carman et al. (2015) (present study)

n.r. not reported

circulating ADMA levels are not considerably different between young diabetics and non-diabetics (Jehlicka et al. 2009; Glowinska-Olszewska et al. 2010) (Table 7).

Significantly higher circulating ADMA concentrations were found in young adults with diabetes mellitus (mean age, 28 years; mean HbA_{1c} of 8.6 %) without cardiovascular complications (Altinova et al. 2007). In healthy children, there are developmental changes in ADMA, i.e., a decrease of ADMA plasma concentration from birth (about 1000 nM) to the age of 16 (about 400 nM) (Lücke et al. 2007). It has been hypothesized that circulating ADMA concentration increases before the appearance of diabetic-dependent cardiovascular complications (Altinova et al. 2007). However, others reported even a significant decrease in circulating ADMA levels in young diabetics with a mean age of 12 years and mean HbA_{1c} of 8.2 % (Huemer et al. 2011). Comparably higher ADMA concentrations were also found in children with renal diseases (Lücke et al. 2008; Brooks et al. 2009; Wang et al. 2007), in hypercholesterolemia (Hasanoğlu et al. 2011), as well as in arterial hypertension (Sladowska-Kozłowska et al. 2012) and pulmonary hypertension (Sanli et al. 2012). The children examined in the present study did not have elevated concentrations of creatinine, cholesterol and triglycerides; also, they had no microalbuminuria, arterial hypertension or pulmonary hypertension (Table 1).

In confirmation of the observation of our previous study in healthy children and adolescents (Lücke et al. 2007), we found in the present study a negative correlation between age and ADMA in the HC group. The age-dependent decline of circulating ADMA concentration was even more evident in the T1DM-T and T1DM-ND groups. This observation and the age-dependent decrease of urinary ADMA and DMA, the major urinary metabolite of ADMA in humans (Tsikas 2008), in the T1DM patients of the present study, suggest that whole body ADMA synthesis decreases with age in humans. In contrast to ADMA, the concentration of circulating hArg increased with age in the T1DM-T group. Consequently, the antidromic developmental changes of hArg and ADMA resulted in age-dependent increase of the hArg/ADMA molar ratio in this group. Provided hArg and ADMA are antagonists in the renal and cardiovascular systems (Tsikas et al. 2014), enhancement of the hArg/ADMA molar ratio in T1DM patients could represent a new strategy to protect young diabetic patients from future cardiovascular disease and related complications. This idea is supported by the observation of the gradual increase of the median hArg/ADMA ratio from 2.70 in T1DM-ND and 2.94 in T1DM-T to 3.13 in HC, albeit not statistically significant.

In addition, there are further factors that have to be considered in this discussion. One of these factors is the disease duration. The children and adolescents who took part in the present study had an average disease duration of 5 years. Perhaps, this period of time is too short for major changes in ADMA and hArg synthesis and/or metabolism. Other important factors to be considered in the discussion are the influence of renal function, glucose or insulin on the metabolism and elimination of ADMA (Vallance et al. 1992; Cighetti et al. 2009; Lajer et al. 2009; Tarnow et al. 2004). Like in adults, the circulating ADMA concentration has been shown to be elevated in children when renal function was impaired (Wang et al. 2007; Lücke et al. 2008; Brooks et al. 2009).

In patients with critical illness, hyperglycemia leads to increased circulating ADMA levels, and maintenance of normoglycemia has been reported to increase NO synthesis (Ellger et al. 2008). In our study, children in the T1DM-T group had a relatively narrow range of HbA_{1c}, indicating fairly good blood glucose control during the previous 6 weeks (Cerami et al. 1979). Acute variations of blood glucose have been reported not to change ADMA levels, unlike insulin which has been found to significantly lower ADMA levels (Marcovecchio et al. 2008). Circulating ADMA levels are unlikely to be influenced by acute variations of blood glucose. Long-term adjustment of blood glucose levels seems to be of higher importance. This might explain why circulating ADMA levels did not differ between the T1DM-T and HC groups in our study.

In the T1DM-T and T1DM-ND groups of the present study, we found opposite relationships between plasma ADMA concentration and HbA_{1c} levels. ADMA and HbA_{1c} correlated positively in the T1DM-ND group, but negatively in the T1DM-T group. The opposite relationships suggest that treatment of newly diagnosed children with T1DM with insulin would reverse the relationship between ADMA and HbA_{1c}. The underlying mechanisms are unknown and remain to be resolved. It is worth mentioning that human erythrocytes are rich in ADMA-containing proteins (not including hemoglobin), the proteolysis of which may release considerable amounts of free ADMA into the circulation (Böhmer et al. 2012; Davids et al. 2012; Grosskopf et al. 2012).

Nitrite and nitrate

In previous studies, we found that renal carbonic anhydrase (CA) was involved in the reabsorption of nitrite (Chobanyan-Jürgens et al. 2012b) and that this nitrite-dependent CA activity could be inhibited by endogenous and exogenous factors, including drugs such as acetazolamide and *N*-acetyl-L-cysteine (Tsikas et al. 2014). This is of particular importance because nitrite is considered an abundant NO reservoir, and proteins such as hemoglobin, xanthine oxidoreductase and CA (Aamand et al. 2009) may convert nitrite to NO.

With respect to the NO metabolites nitrite and nitrate in plasma and urine, we observed that the Arg/NO pathway is altered in children with T1DM. The concentrations of nitrite and nitrate in urine and plasma were significantly lower in diabetic compared to non-diabetic children. These observations suggest that NO synthesis and bioavailability are diminished in children with T1DM. In adults, diminished NO synthesis and bioavailability are generally assumed to be associated with impaired endothelium-dependent vasodilation. In our study, we did not perform specific studies such as flow-mediated dilation experiments to investigate potential impairment of endothelium-dependent vasodilation. Yet, the clinical data of our T1DM patients do not argue for such impairment.

Renal CA isoforms are involved in the reabsorption of nitrite (Chobanyan-Jürgens et al. 2012b). The relative excretion of nitrite to nitrate in urine, i.e., the urinary nitrate-to-nitrite molar ratio ($U_{NOx}R$), may be a useful measure of nitrite-dependent renal CA activity. We found that $U_{NOx}R$ was much lower in elderly adults with T2DM compared to non-diabetic elderly subjects (Tsikas et al. 2015). Interestingly, in our diabetic children under long-term insulin treatment and in newly diagnosed T1DM, the $U_{NOx}R$ values were higher than in HC. The difference in the $U_{NOx}R$ values between elderly T2DM patients (approximately 75) (Tsikas et al. 2015) and the children with T1DM of the present study (approximately 1200) is remarkable. The different type of diabetes and the large difference in age may have contributed to the 16-fold higher $U_{NOx}R$ in children with T1DM and adolescents compared to adults with T2DM. It should also be considered that the Arg/NO pathway in childhood and adolescence differs from that in adults with respect both to Arg and NO metabolites (Lücke et al. 2007). The urinary excretion of nitrite is of the same order of magnitude in T1DM-ND, T1DM-T and HC and healthy adults. The large difference in the $U_{NOx}R$ values likely results from the considerably higher nitrate excretion in T1DM. Another factor that may have contributed to the higher $U_{NOx}R$ values in children with T1DM of our study is that both children with T1DM-ND and T1DM-T have almost three times lower plasma concentrations of nitrite than HC. We should therefore consider in this discussion the $P_{NOx}R$, i.e., the plasma nitrate-to-nitrate molar ratio. The average $P_{NOx}R$ values are comparable in the T1DM-T and T1DM-ND groups (i.e., 69 vs 68) and considerably lower than the $P_{NOx}R$ value in the HC group (i.e., 20). The approximate mean ratios of $U_{NOx}R$ to $P_{NOx}R$ (i.e., $U_{NOx}R/P_{NOx}R$) are calculated to be 17 in T1DM-T, 20 in T1DM-ND and 27 in the HC group. In 18 non-medicated healthy young men (mean age, 27 years) of a previous study (Keimer et al. 2003), we determined $U_{NOx}R/P_{NOx}R$ ratios of 12.4 (7.6–35) and 13.8 (7.2–20) on 2 days with an in-between interval of 3 weeks. The $U_{NOx}R/P_{NOx}R$ values in the young adults differ from those in the HC of the present study and indicate that the nitrite-dependent renal CA activity differs between children/adolescents and adults. On the basis of these data, one may conclude that the nitrite-dependent renal CA activity is altered in T1DM in childhood and adolescence.

Possible study limitations

The concentration of some biochemical parameters of the Arg/NO pathway in blood and urine such as nitrite and nitrate may be influenced by many factors, notably nutrition and physical exercise. Their value regarding the status of the Arg/NO pathway is therefore limited (Tsikas 2015). Such a limitation may also apply to ADMA, because regular physical exercise has been reported to normalize ADMA levels in diabetic patients along with improvement of glycemia (Mittermayer et al. 2005). The differences seen between the T1DM-T and T1DM-ND groups in the present study should be treated with caution in view of the very small size of newly diagnosed children with T1DM included in the study and the considerable difference compared to the T1DM-T group. The Arg/NO pathway in childhood and adolescence has been increasingly investigated. Yet, in comparison to adults, information about the Arg/NO pathway in children is scarce. The age dependence of the status of the Arg/NO pathway represents a challenge and needs to be considered by including a proper and sufficiently high population sample. Our study provides useful information about several players of the Arg/NO pathway including ADMA and hArg. However, understanding the Arg/NO pathway in healthy children and adolescents and those suffering from certain disease(s) including T1DM demands further longitudinal investigations.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The study was designed as a prospective cross-sectional study and was approved by the Ethics Committee of the University of Bochum. The study was performed in accordance with the guidelines of the Declaration of Helsinki and of Good Clinical Practice. Written and informed consent was given by parents and children.

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