Amino Acids (2015) 47:1853–1863
DOI 10.1007/s00726-015-2018-x

ORIGINAL ARTICLE

The L-arginine/NO pathway and homoarginine are altered in Duchenne muscular dystrophy and improved by glucocorticoids

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Received: 31 March 2015 / Accepted: 27 May 2015 / Published online: 12 June 2015
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Abstract The L-arginine/nitric oxide (L-Arg/NO) pathway regulates endothelial function and may play an important role in the pathogenesis of Duchenne muscular dystrophy (DMD). Yet, this pathway is poorly investigated in children suffering from DMD. Endothelial dysfunction can affect the perfusion of contracting muscles, thus leading to ischemia and hypoxia. In the present study, we tested the hypothesis that reduced NO production due to elevated synthesis of N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA), an endogenous inhibitor of NO synthesis, is a possible pathophysiological mechanism for progressive intramuscular muscle ischemia and disturbed endothelial function in children with DMD. Given the possible antagonistic action of homoarginine (hArg) on ADMA, we also analyzed this amino acid. We investigated 55 male patients with DMD and 54 healthy male controls (HC; aged 11.9 ± 4.8 vs. 11.1 ± 4.9 years, mean ± SD). Urinary creatinine and metabolites of the L-Arg/NO pathway were measured in plasma and urine by GC–MS or GC–MS/MS. Urine levels of ADMA and its major urinary metabolite dimethylamine (DMA), nitrite and nitrate ($P < 0.001$ for all) and hArg ($P = 0.002$) were significantly higher in DMD patients compared to HC, while the urinary DMA/ADMA molar ratio was lower ($P = 0.002$). In plasma, nitrate ($P < 0.001$), hArg ($P = 0.002$) and the hArg/ADMA ratio ($P < 0.001$) were lower in DMD than in HC. In plasma, ADMA (631 ± 119 vs. 595 ± 129 nM, $P = 0.149$), arginine and nitrite did not differ between DMD and HC. In DMD, positive correlations between ADMA, DMA or nitrate excretion and the stage of disease (according to Vignos and Thompson) were found. In DMD patients on steroid medication, lower concentrations of ADMA in plasma, and of DMA, ADMA, nitrate and hArg in urine were observed compared to non-treated patients. The L-Arg/NO pathway is impaired in DMD patients, with the disease progression being clinically negatively correlated with the extent of impairment. One of the underlying mechanisms in DMD may involve insufficient antagonism of ADMA by hArg. Steroids, but not creatine supplementation, seems to improve the L-Arg/NO pathway in DMD.

Keywords ADMA · Children · Duchenne muscular dystrophy · Homoarginine · Nitric oxide · Steroids

Abbreviations

ACE	Angiotensin-converting enzyme
ADMA	Asymmetric dimethylarginine (N^G,N^G-dimethyl-L-arginine)
AGAT	Arginineglycine amidinotransferase
DDAH	Dimethylarginine dimethylaminohydrolase
DMA	Dimethylamine
DMD	Duchenne muscular dystrophy
EDRF	Endothelium-derived relaxing factor
GAMT	Guanidinoacetate *N*-methyltransferase
GC–MS	Gas chromatography–mass spectrometry
GC–MS/MS	Gas chromatography–tandem mass spectrometry
hArg	Homoarginine
NO	Nitric oxide

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NOS	Nitric oxide synthase
SDMA	Symmetric dimethylarginine (N^G,$N^{G'}$-dimethyl-L-arginine)
sVCAM-1	Soluble vascular cell adhesion molecule-1

Introduction

Duchenne muscular dystrophy (DMD) is a dystrophinopathy which is inherited in an X-linked recessive manner with an incidence of about 1:3.500–6.000 in newborn boys. The disease is a result of mutations in the dystrophin gene localized in the short arm of chromosome Xp21 (Koenig et al. 1987; Annexstad et al. 2014; Mortier 1994), leading to an absence or a significant loss of functional dystrophin (Aartsma-Rus et al. 2006; Annexstad et al. 2014). Dystrophin is a structural protein and part of the dystrophin–glycoprotein complex stabilizing the sarcolemma. As a result of functional loss of dystrophin, the patients suffer from a progressive damage of muscle fibers accompanied by an increasing muscle weakness (Annexstad et al. 2014; Arahata et al. 1988). The progression of the disease shows a wide range of intra-individual differences. Thompson and Vignos established a staging (stage 0–11) for DMD regarding ambulatory ability. Stage 0 describes a patient with DMD without any clinical symptoms, while a patient at stage 11 is bedfast and not even able to sit (Thompson and Vignos 1959; Mortier 1994). DMD cannot be cured; however, a therapy with glucocorticoids can decelerate the progression of muscle weakness and even enhance muscle strength (Manzur et al. 2008; Annexstad et al. 2014). Also, administration of creatine to DMD patients has been reported to improve muscle strength in short and medium term in muscular dystrophies (Kley et al. 2013; Banerjee et al. 2010; Tarnopolsky et al. 2004).

In the 1980s, the endothelium-derived relaxing factor (EDRF) was found to be responsible for the relaxation of vascular smooth muscles by acetylcholine (ACh). EDRF was then identified as nitric oxide (NO) (Moncada and Higgs 2006; Furchgott et al. 1984; Furchgott and Zawadzki 1980; Palmer et al. 1987). NO is generated from L-arginine by oxidation of one of its terminal guanidine groups. This reaction is catalyzed by nitric oxide synthase (NOS; Palmer et al. 1988; Marletta 1993). In humans, three different isoenzymes of NOS are known, which have initially been named after the tissue in which they have been found at first: the constitutive neuronal NOS (nNOS or NOSI), endothelial NOS (eNOS or NOSIII) and the inducible NOS (iNOS or NOSII). iNOS is especially expressed in macrophages and other cells of the immune system (Förstermann et al. 1994; Moncada and Higgs 2006).

In human organisms, NO takes part in many different physiological and pathological processes. In the vascular system, NO is responsible for regulation of blood pressure by dilating vessels and for inhibition of platelet aggregation. Furthermore, NO prevents leukocyte adhesion to the blood vessel wall and vascular smooth muscle proliferation, so that NO protects against atherosclerosis (Moncada and Higgs 2006; Förstermann et al. 1994; Rees et al. 1989). In this regard, NO seems to be an important signaling molecule avoiding different cardiovascular diseases. Many of these diseases have their origin in an endothelial dysfunction due to a decrease of endothelial NO synthesis/bioavailability (Moncada and Higgs 2006). As direct measurement of authentic NO is very difficult in human biological samples including blood, nitrate and nitrite in blood and urine are determined and used for evaluating NO synthesis in vivo (Tsikas 2005).

The activity of NOS is controlled by endogenous inhibitors, among which N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) is the most important one (Leiper and Vallance 2006). After being released in blood circulation, ADMA is partially eliminated unchanged by the kidneys. About 90 % of daily produced ADMA is first hydrolyzed to L-citrulline and dimethylamine (DMA) by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). DMA is then excreted in the urine by the kidneys (Leiper and Vallance 2006; Achan et al. 2003). In adults, there are several cardiovascular, renal and other endothelial dysfunction-associated diseases, in which elevated ADMA concentrations and lower concentrations of nitrate and nitrite prevail compared to healthy subjects (Cooke 2000; Sibal et al. 2010). Elevated ADMA synthesis and consequently reduced NO synthesis are probably responsible for the development of atherosclerosis (Kielstein et al. 1999). Currently, there is feverish research on L-homoarginine (hArg) which is a homolog of L-arginine and a non-essential, non-proteinogenic cationic amino acid formed from L-lysine in the kidney (Ryan and Wells 1964; Ryan et al. 1968). hArg has been reported to be a substrate for NOS as well (Moali et al. 1998) and may therefore affect NO-dependent endothelial function (Valtonen et al. 2008). Low concentrations of hArg are positively associated with cardiovascular and all-cause mortality as well as cardiovascular diseases in humans (März et al. 2010; Pilz et al. 2011, 2014, 2015).

In recent years, the role of ADMA and other members of the L-Arg/NO pathway in children with renal and metabolic diseases has been investigated (Goonasekera et al. 1997; Lücke et al. 2006a, b, 2007, 2008; Kanzelmeyer et al. 2012, 2014). Elevated ADMA levels were found for example in children with hypertension (Goonasekera et al. 1997) and citrullinemia (Lücke et al. 2006a). Moreover, the developmental changes in the L-Arg/NO pathway from infancy to adulthood have been investigated (Lücke et al. 2007). Thus, ADMA levels in plasma were found to decrease with age,

so that ADMA concentrations in young healthy controls are much higher than in healthy adults, while adolescents almost reach the levels prevailing in adulthood (Lücke et al. 2007).

Up to now, there has been little reported data on the L-Arg/NO pathway in children with Duchenne muscular dystrophy, even though it seems to play an important role in the pathogenesis of DMD. The dystrophin complex was found to interact with the N-terminal domain of nNOS, so that the loss of dystrophin in patients with DMD leads to an absence of nNOS in skeletal muscle sarcolemma (Brenman et al. 1995). nNOS is highly expressed in mammalian skeletal muscle and NO produced by nNOS diminishes the sympathetic vasoconstriction in contracting skeletal muscles, so that blood flow is increased. This mechanism does not function in the mdx mouse which is a model of DMD with dystrophin deficiency and resulting loss of nNOS in skeletal muscles. It was supposed that the unopposed sympathetic vasoconstriction leads to progressive ischemia, hypoxia and destruction of skeletal muscles in DMD (Thomas et al. 1998; Chavoshan et al. 2002). In recent years, the NO pathway in DMD was investigated in a small number of studies. It was reported that circulating nitrite/nitrate levels in patients with DMD are lower than those in healthy controls (Gücüyener et al. 2000; Kasai et al. 2004).

In the present study, we tested the hypothesis that reduced NO synthesis due to elevated ADMA concentrations antagonizing NOS would be a possible pathophysiological mechanism for disturbed endothelial function and impaired vessel reactivity in children with DMD. Given the emerging importance of hArg in the cardiovascular system (März et al. 2010; Pilz et al. 2011, 2014, 2015) and its possible antagonistic action on ADMA (Tsikas and Kayacelebi 2014), we included circulating and urinary hArg in our investigations.

Materials and methods

Subjects

We investigated 55 male patients with DMD and 54 healthy male controls aged between 1 and 23 years. Patients with DMD were recruited during routine examinations taking place every 6 months in the Departments of Neuropediatrics of the University Children's Hospitals in Bochum and Essen. Healthy controls were subjects undergoing little surgical operations in the Hannover Medical School and elective gastroscopies in the Children's Hospital in Bochum. The study was designed as a cross-sectional pilot study with the intention to investigate the L-Arg/NO pathway in children with DMD. The Ethics Committees of the Faculty of Medicine at Ruhr-University Bochum and of the Hannover Medical School approved the study and written consent was given by each participant and his parents or only by the participant if he was 18 years or older.

DMD was diagnosed by molecular analysis and/or muscle biopsy. Exclusion criteria were ongoing infections, chronic kidney, liver or metabolic diseases, malignancies, epilepsy, ingestion of blood thinner and fish consumption 24 h prior to the examination. Thirty-four of our DMD patients were treated with glucocorticoids (25 with deflazacort and 9 with prednisolone), whereas 21 patients stopped or had never begun this therapy. In addition to this therapy, most of our patients received vitamin D and calcium ($n = 33$), and some patients ($n = 7$) obtained also creatine (1 or 2 g creatine monohydrate per day, without a pause or in some cases for 3 months with a pause of 1 month). Depending on heart affection, 15 of our patients took angiotensin-converting enzyme (ACE) inhibitors or ACE inhibitors plus beta-blockers. One patient suffering from a cardiac decompensation was treated with more than two antihypertensive drugs.

We defined the severity of loss of ambulation by the staging established by Thompson and Vignos (Thompson and Vignos 1959; Mortier 1994). We included three DMD patients supported by non-invasive ventilation at night and one patient fed by percutaneous endoscopic gastrostomy. Moreover, one of our patients was additionally diagnosed with a selective immunoglobulin A deficiency and another one a growth hormone deficiency.

Patients taking part in the present study were not fasting and did not follow a special diet. The only exception was avoidance of fish consumption in the last 24 h for minimizing dietary DMA intake (Tsikas et al. 2007; Tsikas 2008).

The number of investigated patients was different for the biochemical parameters we analyzed, as urine and blood samples were not available for each child. Also, we determined hArg in plasma and urine in the DMD patients and healthy subjects who had been recruited in Bochum and Essen. This information is provided in Table 1 and in the respective places of this article.

Analytical methods

Venous blood was drawn using ethylenediaminetetraacetic acid (EDTA). The blood samples were immediately put on ice, centrifuged (4500×g, 4 °C, 10 min) and the supernatant plasma samples were frozen at −80 °C until assay. In general, the time point of blood collection relative to the last meal ranged considerably in our DMD patients, but was not noted down. Urine samples from spontaneous micturition (five times obtained from the urinary bag) were frozen immediately at −20 °C until analysis. Urinary levels of ADMA, DMA, nitrate, nitrite and hArg excretion were corrected for creatinine excretion and expressed as μmol of

Table 1 Demographic, clinical and biochemical characteristics of the studied children with DMD and healthy controls

	Healthy children		DMD patients		*P* value
	n	Mean ± SD	*n*	Mean ± SD	
Number of patients in total		54		55	
Age (years)	54	11.1 ± 4.9	55	11.9 ± 4.8	0.387
Height (cm)	39	149 ± 30	53	130 ± 28	0.010
Weight (kg)	39	45 ± 19	55	42 ± 21	0.658
BMI (kg/m^2)	39	19 ± 3.3	52	22 ± 5.8	0.145
ADMA in plasma (nM)	50	595 ± 129	54	631 ± 119	0.149
ADMA in urine (μmol/mmol creatinine)	45	8.86 ± 12.1	54	20.3 ± 10.2	<0.001
DMA in urine (μmol/mmol creatinine)	38	48.1 ± 47.6	54	78.9 ± 29.2	<0.001
DMA/ADMA molar ratio in urine	38	10.1 ± 20.4	54	4.27 ± 1.19	0.002
Arg in plasma (μM)	51	76.6 ± 20.9	54	75.9 ± 15.7	0.857
hArg in plasma (μM)	16	1.76 ± 0.56	54	1.35 ± 0.59	0.002
hArg in urine (μmol/mmol creatinine)	12	0.27 ± 0.18	54	0.98 ± 1.34	0.003
hArg/ADMA ratio in plasma	16	3.44 ± 1.45	54	2.18 ± 0.90	<0.001
Nitrite in plasma (μM)	29	1.51 ± 0.76	53	1.10 ± 0.36	0.154
Nitrate in plasma (μM)	29	38 ± 17.1	53	27.4 ± 8.2	<0.001
Nitrite in urine (μmol/mmol creatinine)	45	0.46 ± 0.95	54	0.97 ± 1.10	<0.001
Nitrate in urine (μmol/mmol creatinine)	45	187 ± 335	54	230 ± 126	<0.001
U_{Nox}R	45	730 ± 591	54	424 ± 353	0.001
Steroid medication	54	N.A.	55	34	N.A.
Creatine supplementation	54	N.A.	55	7	N.A.
Disease stage (Vignos and Thompson) ≥7	54	N.A.	55	28	N.A.
PEG	54	N.A.	55	1	N.A.
Non-invasive ventilation	54	N.A.	55	3	N.A.
ACE inhibitors	54	N.A.	55	6	N.A.
ACE inhibitors + beta-blockers	54	N.A.	55	10	N.A.

N.A. not applicable

the analyte per mmol of creatinine. Urinary creatinine was determined by GC–MS (Tsikas et al. 2010a). ADMA in plasma and urine as well as Arg in plasma was quantitated by gas chromatography–tandem mass spectrometry (GC–MS/MS) and gas chromatography (GC–MS), respectively (Tsikas et al. 2003). Nitrite and nitrate were quantified in plasma and urine simultaneously by GC–MS (Tsikas 2000). DMA in urine was determined by GC–MS (Tsikas et al. 2007). hArg in plasma and in urine was measured by GC–MS/MS (Kayacelebi et al. 2014a, 2015).

Quality control (QC) samples were analyzed alongside study samples as described previously for each analyte and biological sample. All biochemical parameters were determined in the QC samples with accuracy (bias, %) and imprecision (relative standard deviation, %) of less than ±20 and ≤20 %, respectively, indicating the validity of the analytical results in the plasma and urine samples of the study.

Statistical analysis

We used the Shapiro–Wilk and the Kolmogorov–Smirnov normality tests to evaluate data distribution. The data of our two groups (patients with DMD and healthy controls) were compared using the non-parametric Mann–Whitney test for not normally distributed data and the Student's *t* test for unpaired samples for normally distributed parameters. Data are presented as mean ± standard deviation (SD). Spearman's correlation coefficient was applied to assess correlations between the parameters and the stage of disease, according to Vignos and Thompson. Student's *t* test (Mann–Whitney test for non-parametric variables) was also used for intergroup analysis of patients treated and not treated with steroids and creatine. For the comparison of two groups, a *P* value of <0.05 was considered statistically significant. All calculations were executed using the SPSS package (version 20, IBM Corp., Armonk, NY, USA).

Table 2 Biochemical characteristics of DMD patients without and with steroid medication

	Steroid medication	No steroid medication	*P*
ADMA in plasma (nM)	600 ± 119	680 ± 104	0.015
ADMA in urine (µmol/mmol creatinine)	15.7 ± 7.95	28.1 ± 8.77	<0.001
DMA in urine (µmol/mmol creatinine)	65.1 ± 21.7	102 ± 25.4	<0.001
Nitrate in urine (µmol/mmol creatinine)	188 ± 72.5	302 ± 162	0.001
hArg in urine (µmol/mmol creatinine)	0.53 ± 0.47	1.75 ± 1.92	0.001
DMA/ADMA molar ratio in urine	4.55 ± 1.27	3.79 ± 0.85	0.02

Table 3 Biochemical characteristics of DMD patients without and with creatine supplementation

	Creatine supplementation	No creatine supplementation	*P*
hArg in urine (µmol/mmol reatinine)	0.79 ± 0.78	1.01 ± 1.41	0.887
hArg in plasma (µM)	1.20 ± 0.44	1.37 ± 0.61	0.403
hArg/ADMA ratio in plasma	2.18 ± 0.78	2.18 ± 0.93	0.990
ADMA in plasma (nM)	561 ± 121	641 ± 117	0.078
Arg in plasma (µM)	67.9 ± 7.3	77.1 ± 16.4	0.062
DMA in urine (µmol/mmol creatinine)	58.4 ± 30.9	81.9 ± 28.0	0.036
Nitrate in urine (µmol/mmol creatinine)	160 ± 60	241 ± 130	0.046

Results

We measured several biochemical parameters of the L-Arg/NO pathway in plasma and urine samples of 55 male patients with DMD and 54 healthy male controls by GC–MS or GC–MS/MS methods. Yet, biological samples were not available from all children. The results of the present study are summarized in Tables 1, 2, 3 and Fig. 1.

ADMA in plasma (631 ± 119 vs. 595 ± 129 nM, $P = 0.149$) was higher in DMD patients than in healthy controls. Urine levels of ADMA ($P < 0.001$), DMA ($P < 0.001$), nitrite ($P < 0.001$), nitrate ($P < 0.001$) and hArg ($P = 0.003$) were significantly higher in DMD patients than in healthy controls. Regarding urinary ADMA, our observations confirm the increased urinary excretion of ADMA in DMD patients (Inoue et al. 1979). The DMA/ADMA molar ratio in urine ($P = 0.002$), nitrate in plasma ($P < 0.001$), hArg in plasma ($P = 0.002$) and the hArg/ADMA ratio in plasma ($P < 0.001$) were significantly lower in DMD patients than in healthy patients. There was no statistically significant difference between plasma Arg, plasma nitrite and plasma ADMA in DMD patients and healthy controls.

In DMD patients, there was a positive correlation between the stage of disease and urine DMA ($r = 0.522$, $P < 0.001$; Fig. 1d), urine ADMA ($r = 0.652$, $P < 0.001$; Fig. 1e) or urine nitrate ($r = 0.507$, $P < 0.001$; not shown). In contrast, the DMA/ADMA molar ratio in urine correlated negatively with the stage of disease ($r = -0.566$, $P < 0.001$; Fig. 1f). No significant dependence on the stage of disease was found for hArg (Fig. 1a), ADMA (Fig. 1b), hArg/ADMA ratio (Fig. 1c) in plasma, for nitrite in urine, nitrite in plasma and nitrate in plasma (data not shown).

In the DMD patients on steroid medication (Table 2), we measured significantly lower plasma ADMA concentrations (600 ± 119 vs. 680 ± 104 nM, $P = 0.015$) compared to patients not medicated with steroids. In the DMD patients on steroid medication, urinary ADMA (15.7 ± 7.95 vs. 28.1 ± 8.77 µmol/mmol creatinine, $P < 0.001$), DMA (65.1 ± 21.7 vs. 102 ± 25.4 µmol/mmol creatinine, $P < 0.001$), nitrate (188 ± 72.5 vs. 302 ± 162 µmol/mmol creatinine, $P = 0.001$) and hArg (0.53 ± 0.47 vs. 1.75 ± 1.92 µmol/mmol creatinine, $P = 0.001$) were lower than in patients not medicated by steroids. The DMA/ADMA molar ratio in urine was significantly higher in DMD patients on steroid medication compared to DMD patients without steroid medication (4.55 ± 1.27 vs. 3.79 ± 0.85, $P = 0.02$).

In previous studies, creatine supplementation was found to alter the plasma concentration of guanidino compounds including guanidinoacetate (Derave et al. 2004), the precursor of creatine. We compared the different biochemical parameters of the seven DMD patients being on creatine supplementation with those of DMD patients not treated with creatine (Table 3). hArg in urine (0.79 ± 0.78 vs. 1.01 ± 1.41 µmol/mmol creatinine, $P = 0.887$), hArg in plasma (1.20 ± 0.44 vs. 1.37 ± 0.61 µM, $P = 0.403$) and the plasma hArg/ADMA ratio (2.18 ± 0.78 vs. 2.18 ± 0.93, $P = 0.990$) did not differ between the two groups. Nearly or statistically significantly lower concentrations upon creatine treatment were obtained for ADMA (561 ± 121 vs. 641 ± 117 nM, $P = 0.078$) and Arg (67.9 ± 7.3 vs. 77.1 ± 16.4 µM, $P = 0.062$) in plasma, and for DMA

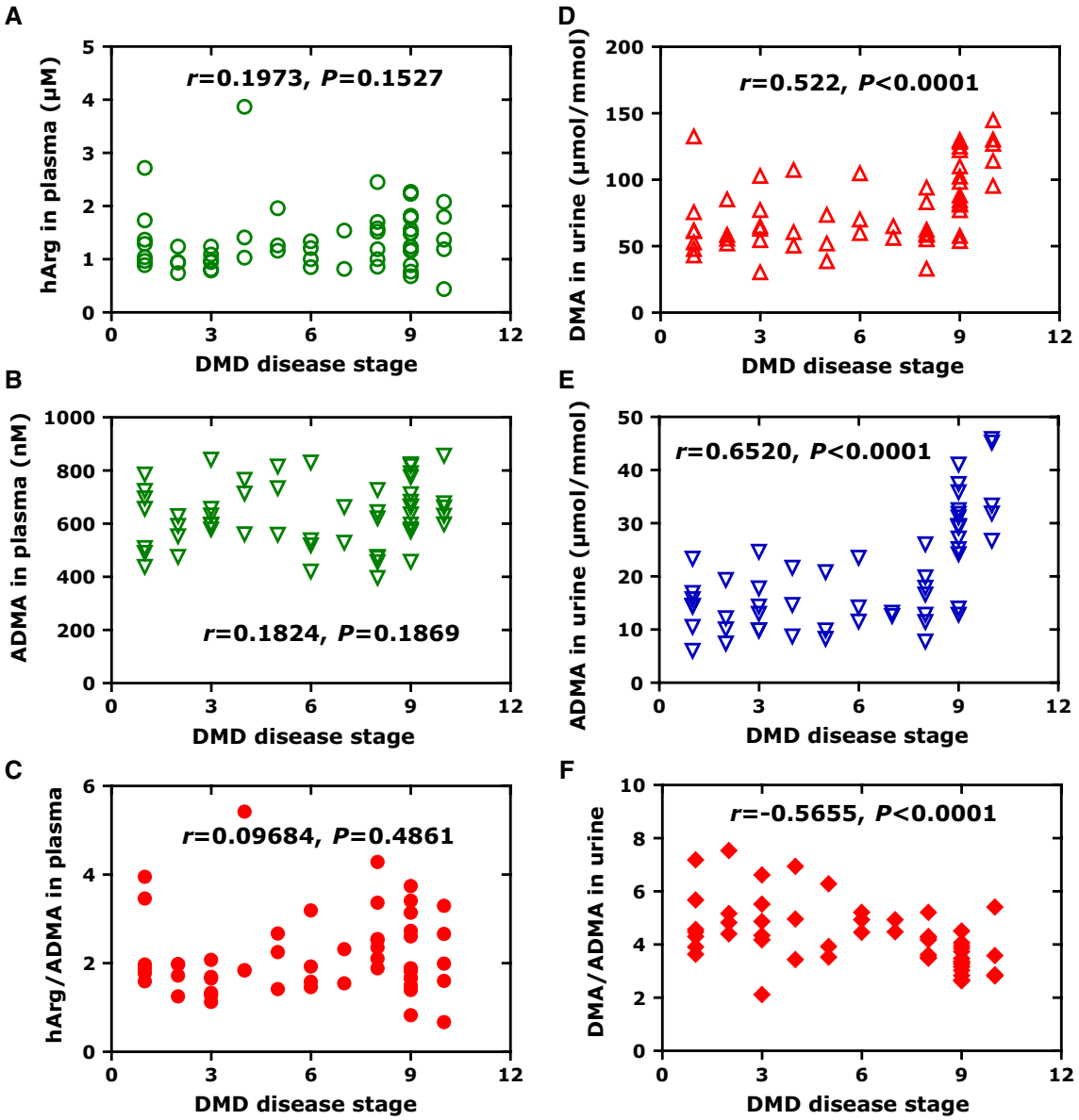

Fig. 1 Spearman's correlation between stage of disease according to Vignos and Thompson and **a** hArg in plasma, **b** ADMA in plasma, **c** hArg/ADMA ratio in plasma, **d** urinary DMA ($r = 0.522$, $P < 0.001$), **e** urinary ADMA ($r = 0.652$, $P < 0.001$) and **f** DMA/ADMA ratio in urine ($r = -0.566$, $P < 0.001$) in children with DMD

(58.4 ± 30.9 vs. 81.9 ± 28.0 µmol/mmol creatinine, $P = 0.036$) and nitrate (160 ± 60 vs. 241 ± 130 µmol/mmol creatinine $P = 0.046$) in urine.

Discussion

In recent years, the L-Arg/NO pathway in childhood has been increasingly investigated. Our groups have investigated this pathway in healthy children and in children with renal and metabolic diseases (Lücke et al. 2006a, b; Kanzelmeyer et al. 2012, 2014; Chobanyan-Jürgens et al. 2012a). The L-Arg/NO pathway in children differs from that in adults. Interestingly, ADMA synthesis is higher in children compared to adults, yet without signs of cardiovascular diseases. In healthy humans, ADMA synthesis decreases steadily from infancy to adulthood (Lücke et al. 2007). We were interested to know whether the L-Arg/NO pathway is altered in children with DMD. We hypothesized that many of the symptoms seen in DMD could be due to altered L-Arg/NO pathway. We measured several biochemical parameters of the L-Arg/NO pathway in 55 DMD patients and in 54 healthy children of the same age.

There are four main findings of our study. First, urine excretion of ADMA and its main metabolite DMA are

significantly higher in DMD patients than in healthy controls. The concentration of ADMA in plasma is higher in DMD compared to healthy children, but the difference did not show statistical significance. Second, nitrate in plasma is lower, but nitrate and nitrite excretion are higher in DMD patients compared to healthy controls. Third, the concentration of hArg and the hArg/ADMA molar ratio in plasma are lower in DMD compared to healthy controls. Steroids but not creatine supplementation seems to improve the L-Arg/NO pathway in DMD. These observations are discussed in the sections that follow.

ADMA and NO synthesis in DMD

The higher excretion of ADMA and its main metabolite DMA in the urine of the DMD children suggest that ADMA synthesis is elevated in DMD. The slightly higher urinary excretion of nitrate in DMD suggests that whole body NO synthesis is higher in DMD patients compared to healthy controls of our study. The higher excretion of nitrite, the lower plasma nitrite concentration and the lower nitrate-to-nitrite molar ratio in urine $U_{NOx}R$ in the DMD patients suggest that NO bioavailability in the renal and cardiovascular systems is impaired in DMD. Diminished NO bioavailability could be a possible mechanism for the progressive muscle ischemia and impaired vessel reactivity in DMD patients (Thomas et al. 1998; Chavoshan et al. 2002).

Diseases such as atherosclerosis, hypertension and chronic heart failure are associated with endothelial dysfunction and elevated circulating ADMA concentrations. ADMA inhibits the activity of all NOS isoforms including eNOS and nNOS (Tsikas et al. 2000; Kielstein et al. 2007). Diminished NO synthesis may therefore impair endothelial function (Böger et al. 1997; Cooke 2000; Sibal et al. 2010). In our DMD patients, elevated synthesis and presumably higher cellular ADMA concentrations may also result in endothelial dysfunction which could affect the perfusion of contracting muscles, eventually resulting in ischemia and hypoxia. This has been demonstrated in the *mdx* mouse, a model of DMD with dystrophin deficiency and loss of nNOS in skeletal muscles (Thomas et al. 1998; Chavoshan et al. 2002).

DMD is characterized by muscular wasting and high rates of protein degradation (Inoue et al. 1979; Tran et al. 2003; Warnes et al. 1981). Protein degradation (i.e., proteolysis) is an important source of free Arg and methylarginines including ADMA (Kakimoto and Ankazawa 1970; Tran et al. 2003). In theory, the elevated circulating and excretory ADMA concentrations measured in the DMD patients of our study could be due to increased protein degradation. However, the almost identical plasma Arg levels in DMD and healthy children argue against this possibility. Another important observation that makes proteolysis an unlikely contributor to ADMA in DMD is that the ADMA isomer symmetric dimethylarginine (SDMA, N^G,$N^{G'}$-dimethyl-L-arginine) was found not be elevated in urine of DMD patients (Inoue et al. 1979). As hArg is not proteinogenic, proteolysis cannot have contributed to hArg in the plasma and urine samples of our DMD patients.

Chronic inflammatory processes seem to play an important role in the pathogenesis of DMD. In dystrophic skeletal muscles a part of the progressive muscle damage is caused by activation of inflammatory cells (De Paepe and De Bleecker 2013; Evans et al. 2009; Spencer and Tidball 2001). Inflammatory cytokines may affect the homeostasis of ADMA (Zoccali et al. 2007). Also, the Arg/ADMA ratio is decreased in conditions with increased C-reactive protein and myeloperoxidase activity (van der Zwan et al. 2011). It is, therefore, possible that chronic inflammatory processes have contributed to elevated ADMA synthesis in our DMD patients.

Measurement of nitrate and nitrite in plasma and urine is commonly used to evaluate NO synthesis in vivo (Tsikas 2005). Yet, this is associated with difficulties and the use of these parameters as measures of NO synthesis and bioavailability is limited (Tsikas 2015). Our patients were not on standardized low-nitrate and low-nitrite diet and were not fasting overnight. Therefore, it is possible that dietary factors might have contributed to the elevated concentrations of nitrite and nitrate in urine. Also, 34 of the DMD patients were on glucocorticoid medication which can raise susceptibility to various infections (Cutolo et al. 2008). The comparatively higher nitrate and nitrite excretion in the urine in our DMD patients suggests that NO synthesis is elevated in DMD. The relatively lower plasma concentration of nitrite, which is considered a measure of endothelial NO production and NO bioavailability in the circulation, suggests that NO bioavailability is diminished in DMD. The higher excretion rate of nitrite in DMD compared to healthy controls suggests an impaired reabsorption of nitrite in the kidneys, finally resulting in loss of NO bioavailability mainly in the form of nitrite. In humans, renal carbonic anhydrases are involved in the reabsorption of nitrite in the proximal tubule of the nephron (Tsikas et al. 2010b; Chobanyan-Jürgens et al. 2012b). The urinary nitrate-to-nitrite molar ratio $U_{NOx}R$ is a useful measure of nitrite-dependent carbonic anhydrase activity (Tsikas et al. 2014). In our DMD patients, the $U_{NOx}R$ value was about 40 % smaller than in healthy controls suggesting considerable impairment of nitrite-dependent renal carbonic anhydrase in DMD.

In a previous study, we found that in adult patients with rheumatism the excretion rate of nitrite was elevated and correlated closely with 3-nitrotyrosine, a biomarker of oxidative stress (Pham et al. 2009). Thus, increased nitrite excretion rate in children with DMD may also be associated with enhanced oxidative/nitrosative stress in this disease, which has been reported to be exacerbated in DMD (Terrill et al. 2013).

hArg, ADMA and their relationship in DMD

Low circulating hArg concentrations are associated with cardiovascular and all-cause mortality (März et al. 2010; Pilz et al. 2011, 2014, 2015). The hArg concentration and hArg/ADMA molar ratio in plasma of DMD patients are lower than in healthy children. hArg may serve as a substrate for NOS (Moali et al. 1998) and thus improve endothelial function (Valtonen et al. 2008). Diminished hArg synthesis in DMD may decrease NO synthesis and add to the cardiovascular risk in this disease. Elevated ADMA and diminished hArg concentrations may promote synergistically the development of cardiomyopathy in DMD patients. This is supported by similar findings on hArg and ADMA in Takotsubo cardiomyopathy (Kayacelebi et al. 2014b). There are indications that hArg antagonizes the effects of ADMA in the renal and cardiovascular systems (Tsikas and Kayacelebi 2014). In the DMD patients of our study, the hArg synthesis is impaired and its antagonistic action on ADMA seems to be insufficient, which is expressed in the lower hArg/ADMA molar ratio in plasma compared to healthy controls.

The L-Arg/NO pathway in relation to the DMD stage of disease

The urinary excretion of ADMA and its metabolite DMA, as well as of nitrate, correlated positively with progressive stage of disease (according to Thompson and Vignos; Thompson and Vignos 1959; Mortier 1994) in the DMD patients. Elevated ADMA synthesis, insufficient antagonism by hArg and the diminished NO bioavailability in the DMD patients are likely to have mediated and increased progressively the endothelial dysfunction accompanied by ischemia, hypoxia and destruction of skeletal muscles. DMD patients affected more severely are usually older than less affected DMD patients. This circumstance and the age-dependent decrease of circulating ADMA in healthy children (Lücke et al. 2007) may explain in part the lack of a correlation between plasma ADMA concentration and stage of disease in the DMD patients.

Effect of steroid and creatine medication

Chronic inflammatory processes seem to play an important role in the pathogenesis of DMD (De Paepe and De Bleecker 2013; Evans et al. 2009; Spencer and Tidball 2001). Inflammatory cytokines play also a role in the L-Arg/NO pathways including ADMA synthesis (Zoccali et al. 2007). Elevated serum ADMA levels have been found in several inflammatory diseases which could be explained by a down-regulation of DDAH activity by cytokines as a result of oxidative stress (Ito et al. 1999). Glucocorticoids are anti-inflammatory drugs and used in the treatment of many chronic inflammatory diseases (Barnes 2010). The effects of steroids on the progression of DMD have been investigated by many groups. Administered glucocorticoids seem to improve muscle strength and to delay the development of cardiac and respiratory complications in DMD (Manzur et al. 2008; Annexstad et al. 2014; Leung et al. 2011). DMD patients on steroid medication were found to excrete less nitrate than DMD patients who have not been medicated with steroids. This is likely to have resulted primarily from an inhibition of iNOS expression reported for glucocorticoids (Radomski et al. 1990). It is worth mentioning that iNOS activity is several orders of magnitude higher than that of nNOS and more so of eNOS (Böhmer et al. 2014). Steroids have anti-inflammatory effects (Barnes 2010) and reduce inflammation in DMD patients. In our study, steroid administration seems to diminish inflammation and thus decrease ADMA synthesis. ADMA is a risk marker for cardiovascular disease (Sibal et al. 2010). Inhibition of ADMA synthesis by steroids could explain the attenuated progression of the disease, including reduction of cardiac and respiratory complications in patients suffering from DMD.

Routine administration of creatine to seven of our DMD patients influenced all biochemical parameters of the L-Arg/NO pathway in the same direction, i.e., it decreased their concentration in plasma and urine. Yet, creatine supplementation did not result in statistically significant changes in hArg, ADMA and their molar ratio hArg/ADMA in plasma compared to 47 of our DMD patients who were not treated with creatine. Interestingly, creatine supplementation decreased both DMA and nitrate excretion suggesting inhibition of whole body synthesis of ADMA and NO, respectively. Although not statistically significant, our results seem to confirm previous results showing an inhibitory action of creatine on the activity of the enzyme arginine:glycine amidinotransferase (AGAT) which is involved in the synthesis both of hArg and guanidinoacetate (Walker and Hannan 1976; Roberts and Walker 1985; da Silva et al. 2014), the substrate of guanidinoacetate: *N*-methyltransferase (GAMT) which catalyzes the *N*-methylation of guanidinoacetate to

creatine. However, the effect of creatine on hArg and NO synthesis remains to be investigated.

Combined administration of L-arginine (3 × 2.5 g/d) and metformin (2 × 250 mg/d) for 16 weeks to five ambulatory and genetically confirmed DMD patients (age 7–10 years) was reported to increase cGMP and mitochondrial proteins of complex III and V, as well as to improve motor function and timed walking distances, without any serious side effects (Bonati et al. 2015, an Abstract). Whether the beneficial effects of the combined treatment seen in this are due to an improvement of the L-Arg/NO pathway and are mediated by L-arginine, metformin or both remains to be investigated. It is worth mentioning that metformin administration (1655 mg/d) may enhance ADMA synthesis, but decrease plasma-soluble vascular cell adhesion molecule-1 (sVCAM-1) in patients suffering from type 2 diabetes mellitus and stable coronary artery disease (Kruszelnicka et al. 2015).

Conclusions

Children with DMD have elevated synthesis of ADMA, diminished hArg synthesis and reduced NO bioavailability compared to healthy children. The extent of impairment of the L-Arg/NO pathway correlates positively with the stage of the DMD disease. Administration of steroids in DMD improves the L-Arg/NO pathway including inhibition of ADMA synthesis and exerts positive effects on the progression of the DMD disease. Administration of hArg in DMD to increase the hArg concentration might be an additional therapeutic means aiming at enhancing the antagonistic effects of hArg to ADMA in the circulation and should be investigated in future studies. Creatine supplementation in DMD seems to suppress the L-Arg/NO pathway.

Acknowledgments The authors thank A. Mitschke and M. T. Suchy for excellent laboratory assistance and F. M. Gutzki for performing GC–MS and GC–MS/MS analyses. We are also grateful for the support in recruiting DMD patients from the Department of Neuropediatrics in the University Hospital Essen.

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

Ethical standard The Ethics Committees of the Faculty of Medicine at Ruhr-University Bochum and of the Hannover Medical School approved the study. Written consent was given by each participant and his parents or only by the participant if he was 18 years or older.

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