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Developmental changes in the L-arginine/nitric oxide pathway from infancy to adulthood: plasma asymmetric dimethylarginine levels decrease with age

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Abstract

Background: The L-arginine/nitric oxide (NO) pathway has multiple physiological functions including vasodilation, inhibition of platelet aggregation and neurotransmission. Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of all known NO synthase isoforms, has adverse effects on renal and cardiovascular function in adults. It is unknown whether ADMA might also exert similar effects in younger individuals including infants. Also, reference data for important members of the L-arginine/NO family, notably ADMA and the NO metabolites, nitrite and nitrate, in infancy are lacking.

Methods: In the present study, we investigated the status of the L-arginine/NO pathway in 34 healthy volunteers aged 2 days to 24 years by measuring the concentration of ADMA, nitrite, nitrate and L-arginine in plasma and urine using gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry methods.

Results: We found that ADMA levels in plasma decreased with age (Pearson correlation coefficient $r=-0.619$, $p<0.001$). In contrast, urinary excretion of nitrate ($r=0.471$, $p=0.036$) and nitrite increased with age ($r=0.484$, $p=0.037$).

Conclusions: Our study suggests that in infants ADMA biosynthesis accompanied by an inhibition of NO synthesis is higher than in adults and diminishes considerably with age.

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Keywords: age-dependency; asymmetric dimethylarginine (ADMA); childhood; infancy; nitric oxide (NO); nitric oxide synthase (NOS).

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Introduction

In humans, three subtypes of nitric oxide synthase (NOS) are known: the inducible NOS (iNOS), the endothelial NOS (eNOS) and the neuronal NOS (nNOS) (1). These enzymes oxidize the imino group of the terminal guanidine group of L-arginine to NO, with L-citrulline being the second reaction product (2). NO is involved in the regulation of blood pressure via vasodilatation in the inhibition of platelet aggregation, in the inhibition of platelet and leukocyte adhesion and in neurotransmission (3–5). Anti-proliferative and anti-atherosclerotic effects are attributed to NO (6). In vivo, NO is rapidly oxidized to nitrate and nitrite, which are excreted in urine. Since detection of NO itself in vivo under basal conditions is very difficult (7, 8), nitrate and nitrite in plasma and urine may serve as indirect parameters for estimating NO production in vivo (8).

The activity of NOS is effectively controlled by endogenous inhibitors with the L-arginine analog asymmetric dimethylarginine (ADMA) being the most important (9, 10). ADMA is produced by methylation of protein-associated L-arginine catalyzed by *N*-methyl protein transferases (11). After regular proteolysis, ADMA is released into the circulation. The bulk of endogenously produced ADMA is hydrolyzed to L-citrulline and dimethylamine (DMA) by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (11), which is highly expressed in the liver (12). Both ADMA and DMA are excreted in urine. Rough estimates indicate that approximately 80% of daily produced ADMA is eliminated by the kidney as DMA (13).

In various disorders, notably renal (7, 14–17) and cardiovascular (18–23) diseases, biosynthesis and bioavailability of NO are compromised. Infusion of ADMA in healthy adults has been shown to exert adverse effects on cardiovascular and renal function at blood concentrations similar to those found in patients with cardiovascular pathology [reviewed in ref. (24)]. A reduction of NO synthesis predisposes to atherosclerosis (25). Oral administration of L-arginine has a protective effect with respect to plaque formation (18). The concentration of circulating and excretory members of the L-arginine/NO pathway, notably of ADMA and of nitrate and nitrite, may be a useful prognostic tool in assessing the risk for renal and cardiovascular diseases, especially for the development of atherosclerosis (18, 26).

In the present study, we investigated the L-arginine/NO pathway in healthy infants, children and adolescents, and quantified both in plasma and in urine relevant members of the L-arginine/NO family with

indicator function, as well as L-arginine in plasma, the endogenous precursor of NO.

Materials and methods

Participants

We included in our study 34 healthy volunteers (10 females, 24 males) aged between 2 days and 24 years. The volunteers were admitted to hospital for minor elective surgery (inguinal hernia, circumcision, orchidopexy) and blood was drawn for routine presurgical work-up. Subjects with underlying systemic diseases, such as cardiovascular, oncological, renal or metabolic disorders were excluded. The study was performed in accordance with the Declaration of Helsinki ethical guidelines. It was approved by the Ethics Committee of Hannover Medical School and written informed consent was obtained from the parents or the participants themselves. We obtained 2 mL of blood using EDTA monovettes (Sarstedt, Nümbrecht, Germany), as well as 5 mL of spontaneous urine from each volunteer.

Analytical methods and statistics

All biochemical parameters were analyzed by using previously reported validated methods and by considering analyte-specific features including blood collection and centrifugation, as well as storage of plasma and urine. In addition, quality control was performed as described elsewhere for the individual analytes (27–29). ADMA in plasma and urine, as well as L-arginine in plasma were determined by gas chromatography-tandem mass spectrometry (GC-MS/MS) on a TSQ 7000 quadrupole mass spectrometer (ThermoElectron, Dreieich, Germany), as described elsewhere (27). Nitrate and nitrite in plasma and urine were determined simultaneously by gas chromatography-mass spectrometry (GC-MS) on a DSQ quadrupole mass spectrometer (ThermoElectron, Dreieich, Germany), as described previously (28). Urinary excretion of the biochemical parameters was corrected for creatinine excretion. Urinary creatinine was determined by HPLC, as described recently (29).

Sample size for the single parameters varied due to the limited amount of urine and blood available in young children.

Data are presented as mean (standard deviation, SD) and were compared using the Student t-test for unpaired samples (SPSS, version 13; SPSS Inc., Chicago, IL, USA). Correlation of circulating and excretory biochemical parameters with age was assessed using the Pearson correlation test (SPSS, version 13). A $p<0.05$ value was considered statistically significant.

Results

The mean concentration of ADMA, L-arginine, nitrite and nitrate in plasma and the mean creatinine-corrected urinary excretion of ADMA, nitrite and nitrate of healthy volunteers investigated in the present study are summarized in Table 1. Pearson correlation coefficient (r) obtained from the correlation of each of these biochemical parameters with subject age are reported in Table 1. These correlations are graphically shown for circulating (Figure 1) and excretory (Figure 2) ADMA and nitrate and nitrite.

We found a decrease in ADMA concentration in plasma with increasing age ($r=-0.619$, $p<0.01$; Figure 1A). This finding was accompanied by increases of urinary excretion rates of nitrate ($r=0.471$, $p=0.036$; Figure 2B) and nitrite ($r=0.484$, $p=0.037$; Figure 2C) with increasing age. No significant age-dependent changes were found for nitrate in plasma ($p=0.92$; Figure 1B), nitrite in plasma ($p=0.91$; Figure 1C), L-arginine in plasma ($p=0.90$; data not shown) and ADMA in urine ($p=0.13$; Figure 2A).

Gender differences were not detected in our study. Furthermore, no significant correlation was found between ADMA in plasma and ADMA in urine, ADMA in plasma and nitrate or nitrite in plasma, ADMA in plasma and nitrate or nitrite in urine, ADMA in urine and nitrate or nitrite in plasma, and ADMA in urine and nitrate or nitrite in urine (data not shown).

Discussion

Data in the literature on the concentration in the circulation and in the urine of relevant members of the L-arginine/NO family, e.g., on nitrite and nitrate reflecting NO production rate and ADMA reflecting extent of NOS inhibition, in young healthy subjects are rare and heterogeneous (15–17, 19, 20, 27, 30, 31) (Tables 2 and 3). Also, in most of these studies sample size was very small. In addition, a comprehensive analysis of the above-mentioned members of the L-arginine/NO family is not reported in any of these studies. This lack of data makes it difficult to assess changes of these parameters, and thus of the status of the L-arginine/NO pathway under pathophysiological conditions including inborn diseases, such as citrullinemia (32) or Schimke-immuno-osseous dys-

Table 1 Plasma concentrations and creatinine-corrected excretion rates of the members of the L-arginine/NO pathway measured in the present study and their correlation with age.

	Mean	SD	Pearson correlation coefficient	p-Value	n
ADMA in plasma, nmol/L	708	161	–0.619	<0.01	34
Arginine in plasma, μmol/L	63	31	0.022	>0.05	33
Nitrate in plasma, μmol/L	60	27	–0.021	>0.05	29
Nitrite in plasma, μmol/L	3	1	0.023	>0.05	29
ADMA in urine, μmol/mmol creatinine	13	18	–0.241	>0.05	20
Nitrate in urine, μmol/mmol creatinine	362	525	0.471	<0.05	20
Nitrite in urine, μmol/mmol creatinine	1	1.6	0.484	<0.05	20

NO, nitric oxide; ADMA, asymmetric dimethylarginine.

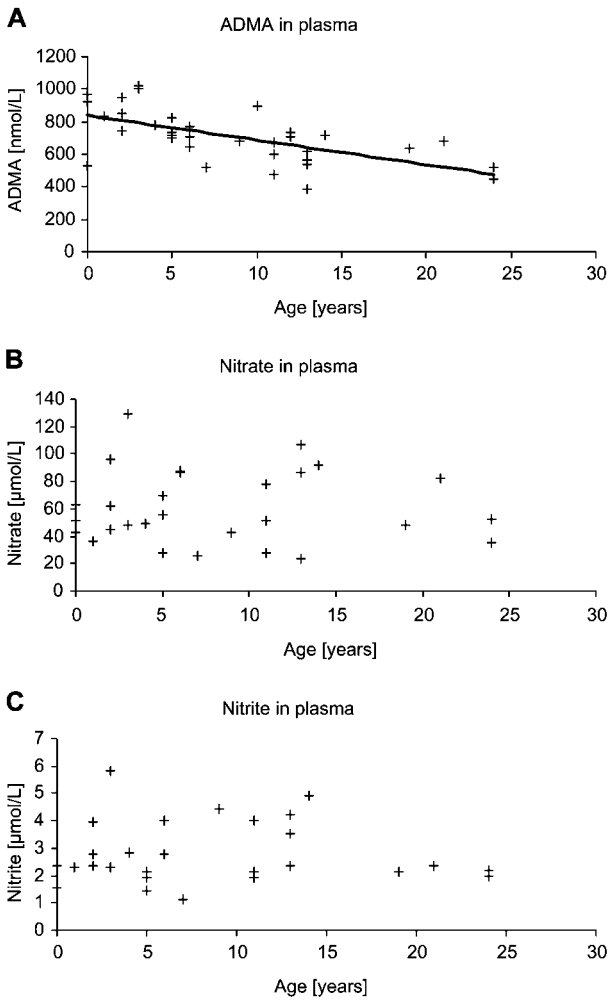

Figure 1 Correlation between plasma concentration of (A) asymmetric dimethylarginine (ADMA) ($n=34$, $r=-0.619$, $p<0.05$), (B) nitrate ($n=29$, $r=-0.021$, $p>0.05$), or (C) nitrite ($n=29$, $r=0.023$, $p>0.05$) and age of healthy infants and young adults.

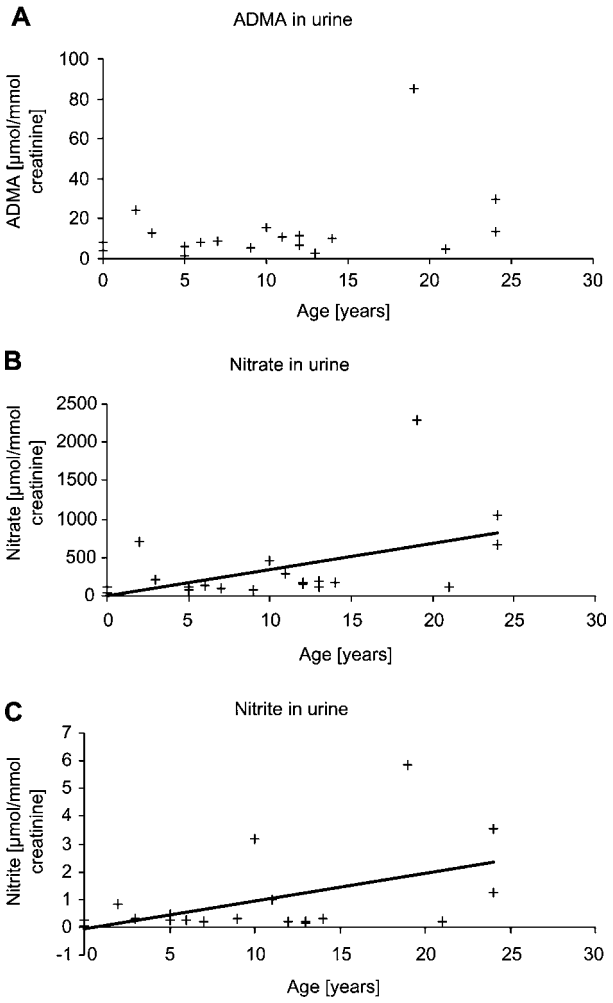

Figure 2 Correlation between urinary creatinine-corrected excretion rate of (A) asymmetric dimethylarginine (ADMA) ($n=20$, $r=-0.241$, $p>0.05$), (B) nitrate ($n=20$, $r=0.471$, $p<0.05$), or (C) nitrite ($n=20$, $r=0.484$, $p<0.05$) and age of healthy infants and young adults.

plasia (33). In our opinion, there is a need to establish comprehensive reference data for infants, children, teenagers and adolescents since the L-arginine/NO pathway is involved in the pathogenesis of different pathological states, e.g., renal (7, 14–17) and cardiovascular (18–23) diseases or hyperlipidemia (26, 34).

In the present study, we investigated the status of the L-arginine/NO pathway in 34 healthy individuals aged 2 days to 24 years by measuring ADMA, L-arginine, nitrate and nitrite both in plasma and in urine using GC-MS/MS and GC-MS techniques. We found that during childhood ADMA levels in plasma decreased considerably from about 1000 nmol/L in infancy to approximately 400 nmol/L in adolescence (Figure 1A). To the best of our knowledge, a correlation between ADMA in plasma and age has not yet been reported in healthy children (aged 2 days to 16 years). The concentration of ADMA in plasma of healthy adolescents (aged 16–24 years) involved in the present study is very close to that reported in the literature (Table 3) (35–37). Interestingly, in healthy adults plasma ADMA concentration has been found to correlate positively with age [reviewed in ref. (35)]. Thus, in a group of 157 healthy adults circulating ADMA concentration correlated with age ($r=0.44$, $p<0.001$); however, ADMA concentration increased from 430 nmol/L for young adults (20–30 years) to only 540 nmol/L for elderly adults (70–80 years) (35). Therefore, numerically the decrease of circulating ADMA concentration by almost 600 nmol/L within the first 20 years of life can be regarded as dramatic in comparison with the increase by only 110 nmol/L of ADMA from 20 to 80 years of age. Nevertheless, small increases in circulating ADMA concentration in adulthood should not be underestimated, because increases of the order of 100 nmol/L in circulating ADMA concentration may be associated with pathologically highly relevant conditions, such as coronary artery disease (38) and other cardiovascular and renal diseases (35–37). Unlike in circulation, ADMA excretion in urine remained almost unchanged over age (Figure 2A). Therefore, it is unlikely that the considerable age-dependent decrease of ADMA in plasma found in our study is due to an increased renal excretion from the postnatal period until adulthood. A more likely explanation could be a decrease of ADMA synthesis, an

Table 2 Summary of reported plasma concentrations and creatinine-corrected excretion rates of members of the L-arginine/NO pathway in healthy children.

	Liappis et al. 1990 (31)	Goonasekera et al. 1997 (19)	Gorenflo et al. 2001 (20)	Elli et al. 2005 (30)	Present study (0–16 years)
ADMA in plasma, nmol/L	n.d.[a]	100 (10)	210 (n.r.[b])	n.d.	725 (160)
Arginine in plasma, μmol/L	36–139	n.d.	n.d.	n.d.	62 (32)
Nitrate in plasma, μmol/L	n.d.	n.d.	n.d.	24.7	62 (28)
Nitrite in plasma, μmol/L	n.d.	n.d.	n.d.	n.d.	3 (1.2)
ADMA in urine, μmol/mmol creatinine	n.d.	n.d.	n.d.	n.d.	9 (6)
Nitrate in urine, μmol/mmol creatinine	n.d.	n.d.	n.d.	147	317 (535)
Nitrite in urine, μmol/mmol creatinine	n.d.	n.d.	n.d.	n.d.	0.9 (1.5)
Number of individuals	n.r.	9	8	296	30
Age range, years	n.r.	1.1–14.4	0.73–12.5	0–16	0–16
Age mean, years	n.r.	8.3	9.3	0–16	6.8 (5)
Method	n.r.	HPLC	HPLC	NO analyzer	GC-MS

[a]n.d., not determined. [b]n.r., not reported. NO, nitric oxide; ADMA, asymmetric dimethylarginine; GC-MS, gas chromatography-mass spectrometry.

Table 3 Summary of reported plasma concentrations and creatinine-corrected excretion rates of members of the L-arginine/NO pathway in healthy children and young adults.

	MacAllister et al. 1996 (15)	Kielstein et al. 1999 (16)	Al Banchaabouchi et al. 2000 (17)	Tsikas et al. 2003 (27)	Present study (16–24 years)
ADMA in plasma, nmol/L	360 (90)	1000 (100)	409 (87)	389 (62)	570 (108)
Arginine in plasma, μmol/L	n.d.[a]	75.5 (3.9)	n.d.	n.d.	71 (22)
Nitrate in plasma, μmol/L	n.d.	39.1 (1.9)	n.d.	n.d.	54 (20)
Nitrite in plasma, μmol/L	n.d.	n.d.	n.d.	n.d.	2.2 (0.2)
ADMA in urine, μmol/mmol creatinine	n.d.	n.d.	n.d.	25.2, 18.4	33 (35)
Nitrate in urine, μmol/mmol creatinine	n.d.	n.d.	n.d.	n.d.	1034 (921)
Nitrite in urine, μmol/mmol creatinine	n.d.	n.d.	n.d.	n.d.	2.7 (2.5)
Number of individuals	9	37	37	12	4
Age range, years	n.r.[b]	n.r.	n.r.	23–64	16–24
Age, years	24 (4)	68.3 (1.1)	64 (14)	35.6	22 (2.4)
Method	HPLC	HPLC	Amino acid analyzer	GC-MS	GC-MS

[a]n.d., not determined. [b]n.r., not reported. NO, nitric oxide; ADMA, asymmetric dimethylarginine; GC-MS, gas chromatography-mass spectrometry.

increase in ADMA metabolism by DDAH or both. To investigate these possibilities, additional measurement of DMA in plasma and urine would be required.

In the present study, the urinary excretion rates of nitrate and nitrite increased with age (Figure 2B, C), reflecting elevation of whole body NO production until adulthood. In line with the results of our present study, we found an increase in urinary excretion of nitrite and nitrate in a subgroup of 7–12-month-old children compared to younger children aged 1–6 months [257 μmol/mmol creatinine vs. 360 μmol/mmol creatinine (39)] in the control group of a previous study comprising 18 age- and sex-matched healthy children, suggesting that in healthy humans NO synthesis starts to increase soon after birth. In contrast, in Zellweger syndrome, a rare peroxisomal disorder, urinary excretion rate of nitrite and nitrate was found to correlate negatively with age in very young children aged 1–12 months in the same study (39). We do not have evidence that alimentary or environmental factors caused the increases of nitrate and nitrite excretion in urine of our healthy young volunteers. It is more likely that the age-dependent increase of NO synthesis, as indicated by increased levels of nitrate and nitrite in urine, is the result of decreased ADMA levels in plasma, which would result in reduced inhibition of all NOS isozymes (10). L-Arginine plasma levels did not show any age-dependency in our healthy young children. Thus, it is unlikely that the diminished NO synthesis in infants as compared to adolescents is caused by an age-dependent decrease in substrate availability for NOS. The design of our study and the biochemical parameters determined do not allow to estimate the activity or expression of individual NOS isoforms. Recently, circulating nitrite has been suggested to mainly reflect eNOS activity in humans (22). In our study, plasma nitrite did not change with age. Should circulating nitrite indeed reflect eNOS activity, the results of the present study would suggest that eNOS activity is rather unaltered from birth until adulthood in healthy humans. Our study supports the hypothesis that the excretion rate of nitrate in the urine is a reliable non-invasive method to assess gradually, long-lasting changes in whole body NO synthesis under basal conditions, as well as upon pharmacological or physical treatment (8).

Children with circulating ADMA concentrations similar to and even higher than those measured in various diseases including renal and cardiovascular

diseases (35–38) are clinically healthy, suggesting that in infancy alternative mechanisms may exist that counteract the inhibitory action of ADMA on NOS. Possible mechanisms could involve participation of potent vasoactive substances from other pathways, such as prostacyclin from the cyclooxygenase pathway. It is worth mentioning that prostacyclin mimics many NO functions, notably vasodilation and inhibition of platelet aggregation. The interplay of the L-arginine/NO pathway with the cyclooxygenase pathway observed previously in the Zellweger syndrome (39) is supportive of such a concept.

In summary, we found that ADMA in plasma fell considerably with age in a group of young healthy individuals and that this fall was accompanied by an increase of nitrate and nitrite excretion in urine. We hypothesize that NOS-antagonism by ADMA is more pronounced in infants compared to young adults. Our findings may be of particular pathophysiological relevance, because in adults a slight elevation of ADMA concentration in plasma has been identified as a risk factor for renal and cardiovascular diseases. Long lasting changes in whole body NO synthesis are best assessed by measuring urinary excretion of nitrate.

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