Amino Acids (2015) 47:1961–1974
DOI 10.1007/s00726-015-2031-0

ORIGINAL ARTICLE

Effects of chronic oral L-arginine administration on the L-arginine/NO pathway in patients with peripheral arterial occlusive disease or coronary artery disease: L-Arginine prevents renal loss of nitrite, the major NO reservoir

Jessica Y. Schneider[1] · Sabine Rothmann[1] · Frank Schröder[1] · Jennifer Langen[2] · Thomas Lücke[2] · François Mariotti[3,4] · Jean François Huneau[3,4] · Jürgen C. Frölich[1] · Dimitrios Tsikas[1]

Received: 27 April 2015 / Accepted: 13 June 2015 / Published online: 28 June 2015

Abstract Despite saturation of nitric oxide (NO) synthase (NOS) by its substrate L-arginine (Arg), oral and intravenous supplementation of Arg may enhance NO synthesis, a phenomenon known as "The L-arginine paradox". Yet, Arg is not only a source of NO, but is also a source for guanidine-methylated (N^G) arginine derivatives which are all inhibitors of NOS activity. Therefore, Arg supplementation may not always result in enhanced NO synthesis. Concomitant synthesis of N^G-monomethyl arginine (MMA), N^G,N^G-dimethylarginine (asymmetric dimethylarginine, ADMA) and N^G,$N^{G'}$-dimethylarginine (symmetric dimethylarginine, SDMA) from supplemented Arg may outweigh and even outbalance the positive effects of Arg on NO. Another possible, yet little investigated effect of Arg supplementation may be alteration of renal function, notably the influence on the excretion of nitrite in the urine. Nitrite is the autoxidation product of NO and the major reservoir of NO in the circulation. Nitrite and Arg are reabsorbed in the proximal tubule of the nephron and this reabsorption is coupled, at least in part, to the renal carbonic anhydrase (CA) activity. In the present placebo-controlled studies, we investigated the effect of chronic oral Arg supplementation of 10 g/day for 3 or 6 months in patients suffering from peripheral arterial occlusive disease (PAOD) or coronary artery disease (CAD) on the urinary excretion of nitrite relative to nitrate. We determined the urinary nitrate-to-nitrite molar ratio ($U_{NOx}R$), which is a measure of nitrite-dependent renal CA activity before and after oral intake of Arg or placebo by the patients. The $U_{NOx}R$ was also determined in 6 children who underwent the Arg test, i.e., intravenous infusion of Arg (0.5 g Arg/kg bodyweight) for 30 min. Arg was well tolerated by the patients of the three studies. Oral Arg supplementation increased Arg (plasma and urine) and ADMA (urine) concentrations. No appreciable changes were seen in NO (in PAOD and CAD) and prostacyclin and thromboxane synthesis (in PAOD). In the PAOD study, $U_{NOx}R$ did not change in the Arginine group (480 ± 51 vs 486 ± 50), but fell in the Placebo group (422 ± 67 vs 332 ± 42, $P = 0.025$). In the CAD study, $U_{NOx}R$ did not change significantly in the Arginine group (518 ± 77 at start vs 422 ± 40 after 3 months vs 399 ± 66 after 6 months), but fell in the Placebo group (524 ± 69 vs 302 ± 36 vs 285 ± 31; $P = 0.025$ for 0 vs 3 months). Infusion of Arg tended to decrease the $U_{NOx}R$ in the children (317 ± 41 vs 208 ± 16, $P = 0.06$). We propose that oral long-term Arg supplementation prevents loss of NO bioactivity by saving nitrite. The optimum Arg dose needs to be elaborated and is likely to be less than 10 g per day in adults. Orally and intravenously administered arginine was well tolerated by the elderly patients and young children, respectively.

Keywords Cardiovascular disease · Nitric oxide · Nitrite · Prostacyclin · Renal carbonic anhydrase · Thromboxane

J. Y. Schneider and S. Rothmann have contributed equally to this work.

✉ Dimitrios Tsikas
Tsikas.Dimitros@mh-hannover.de

[1] Centre of Pharmacology and Toxicology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany

[2] Department of Neuropaediatrics, Children's Hospital, University of Bochum, Bochum, Germany

[3] INRA, CRNH-IdF, UMR914 Nutrition Physiology and Ingestive Behavior, 75005 Paris, France

[4] AgroParisTech, CRNH-IdF, UMR914 Nutrition Physiology and Ingestive Behavior, 75005 Paris, France

Abbreviations

ADMA	Asymmetric dimethylarginine (L-N^G,N^G-dimethylarginine)
Arg	Arginine
CA	Carbonic anhydrase
CAD	Coronary artery disease
cGMP	Cyclic guanosine monophosphate
DBP	Diastolic blood pressure
GC–MS	Gas chromatography–mass spectrometry
GC–MS/MS	Gas chromatography–tandem mass spectrometry
GHD	Growth hormone deficiency
NO	Nitric oxide
NOS	Nitric oxide synthase
PAOD	Peripheral arterial occlusive disease
PGI_2	Prostaglandin I_2 (i.e., prostacyclin)
$P_{NOx}R$	Plasma nitrate-to-nitrite molar ratio
SBP	Systolic blood pressure
TxA_2	Thromboxane A_2
$U_{NOx}R$	Urinary nitrate-to-nitrite molar ratio

Introduction

The semi-essential, proteinogenic amino acid L-arginine (Arg) plays multiple physiological roles (Wu et al. 2009). A quantitatively minor proportion of less than 0.1 % of Arg is converted to L-citrulline (Cit) and nitric oxide (NO) by NO synthase (NOS) isoforms which are expressed in virtually all types of cells (Förstermann and Sessa 2012). NO derived from Arg in endothelial cells by the action of endothelial NOS (eNOS) is one of the most potent endogenous vasodilators and inhibitors of platelet aggregation. Regulation of blood pressure and platelet function are considered the most important roles of NO in human circulation (Moncada and Higgs 1993). NO is autoxidized to nitrite (NO_2^-). NO and NO_2^- are oxidized in erythrocytes by oxyhemoglobin to nitrate (ONO_2^-). After their export into the plasma, NO_2^- and ONO_2^- are excreted in the urine. Circulating and urinary NO_2^- and ONO_2^- may serve under certain conditions as measures of NO synthesis and bioavailability (Tsikas et al. 2006; Tsikas 2015). Some proteins and enzymes such as hemoglobin, xanthine oxidoreductase and carbonic anhydrase (CA) have been reported to reduce nitrite to NO (Lundberg and Govoni 2004; Rassaf et al. 2004; Gladwin et al. 2006; Aamand et al. 2009), independent of NOS. Bacterial nitrate reductases reduce ONO_2^- to NO_2^-. Nutrition and drugs including organic nitrates are also sources of NO_2^- and ONO_2^-. Thus, there is an ONO_2^-/NO_2^-/NO cycle in the body, which maintains a considerable and permanent level of NO bioactivity. Circulating NO_2^- and its companion ONO_2^- are considered a major reservoir of NO (Gladwin et al. 2006; Lundberg et al. 2009).

We have recently shown that renal CA isoforms are involved in the reabsorption of NO_2^- and to a much lesser extent of ONO_2^- (Tsikas et al. 2010a, b; Chobanyan-Jürgens et al. 2012; see also Sütö et al. 1995). This newly discovered role of renal CA activity may be of particular importance, because enhanced excretion of NO_2^- due to diminished reabsorption of NO_2^- or due to inhibition of renal CA activity would result in loss of NO bioactivity. Impaired NO synthesis, particularly in the endothelium, and decreased NO bioavailability in the circulation due to elevated oxidative stress are considered major contributors to the development and progression of renal and cardiovascular diseases and complications in different conditions including diabetes and arteriosclerosis (Tousoulis et al. 2011, 2012; Förstermann and Sessa 2012).

Despite saturation of eNOS by its substrate Arg, supplementation of Arg in form of proteins or as a drug may enhance NO synthesis, a phenomenon known as "The L-arginine paradox" (Tsikas et al. 2000a, b; Böger 2004; Mariotti et al. 2013). Yet, dietary and pharmaceutical Arg is not only a source of NO, but is also a source for guanidine-methylated (N^G) arginine, i.e., N^G-monomethyl arginine (MMA), N^G,N^G-dimethylarginine (asymmetric dimethylarginine, ADMA), and N^G,$N^{G'}$-dimethylarginine (symmetric dimethylarginine, SDMA). MMA, ADMA and SDMA are all inhibitors of eNOS activity (Tsikas et al. 2000a, b; Kielstein et al. 2007; Kayacelebi et al. 2015a, b). Therefore, Arg supplementation may not always result in enhancement of NO synthesis. Concomitant synthesis of the eNOS inhibitors MMA, ADMA and SDMA from exogenous Arg may outweigh and even outbalance the positive effects of Arg as an NO precursor. Another possible, not yet investigated effect of Arg supplementation may be alteration of renal function, notably the influence on the renal excretion of NO_2^-. Interaction of Arg with renal excretion of NO_2^- and ONO_2^- appears possible as the kidney plays a major role in arginine metabolism (Brosnan and Brosnan 2004).

In consideration of the potential role of renal CA isoforms in maintaining NO homeostasis by regulating reabsorption of NO_2^-, we hypothesized that enhanced excretion of NO_2^- in the urine may be an additional, not yet considered factor affecting NO homeostasis in the circulation. Previously, we showed that CA inhibitors such as the strong CA inhibitor acetazolamide (IC_{50}, 100 nM against bovine CAII esterase activity) and the weak CA inhibitors *N*-acetylcysteine (Tsikas et al. 2014) and paracetamol (Innocenti et al. 2008) enhance at therapeutically relevant doses NO_2^- excretion in the urine by inhibiting renal CA activity. In the present human study, we investigated the effects of chronic oral administration of Arg in the verum group (Arginine group) or mannitol in the placebo group

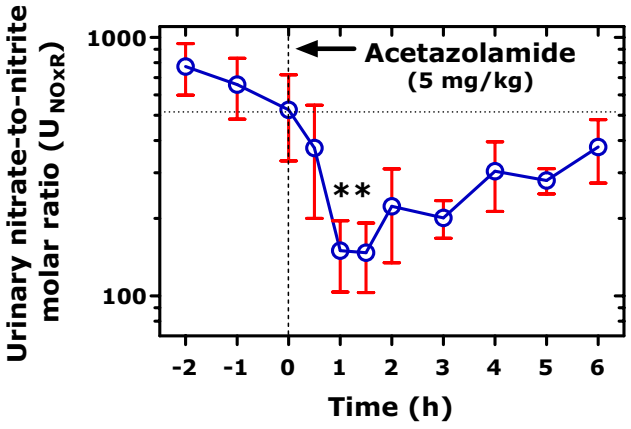

Fig. 1 Urinary nitrate-to-nitrite molar ratio $U_{NOx}R$ (mean ± SEM) in healthy human subjects ($n = 6$) before and after oral administration (5 mg/kg body weight) of the strong CA inhibitor acetazolamide (IC_{50} 0.1 μM) (Chobanyan-Jürgens et al. 2012). The *vertical dotted line* and the *arrow* indicate the time point of drug intake (0 min). This Figure was constructed using the individual nitrate and nitrite excretion data reported elsewhere (Chobanyan-Jürgens et al. 2012). Note the logarithmic scale on the *y*-axis. *Asterisk* statistical significance ($P < 0.05$)

(Placebo group) on various clinical parameters. Our focus was on the urinary nitrate-to-nitrite molar ratio (U_{NOxR}) in patients with peripheral arterial occlusive disease (PAOD) or coronary artery disease (CAD). This is because U_{NOxR} turned out to be a measure of NO bioavailability loss regulated by renal CA (Fig. 1). Thus, oral ingestion of acetazolamide by healthy subjects resulted in an abrupt decrease of U_{NOxR} indicating inhibition of renal CA isoforms. Large amounts of Arg are daily taken up by humans, namely in form of proteins by nutrition (Brosnan and Brosnan 2004). Arg has also been administered to humans in the recent past as a drug, for instance as its hydrochloride salt in effervescent tablets, mainly to increase NO production (reviewed in Bode-Böger 2006). These studies indicated that orally administered Arg of the order of 10 g per day to adults is safe and well tolerated (Bode-Böger 2006; Mariotti et al. 2013). The safety and efficacy of long-term supplementation of Arg have been demonstrated in animal experiments as well (Hu et al. 2015; Yang et al. 2015). In addition to measuring ONO_2^- and NO_2^- in the urine, we also determined these anions in plasma of the PAOD and CAD patients as markers of systemic NO production and NO bioavailability, respectively (Tsikas 2015). The individual nitrate and nitrite concentrations were used to calculate the plasma nitrate-to-nitrite molar ratio (P_{NOxR}). Acute effects of intravenously infused Arg were investigated in children with suspected growth hormone deficiency (GHD) and of orally ingested Arg in form of high-fat protein meal in overweight men.

Methods

PAOD and CAD studies

We performed double-blind, placebo-controlled studies on patients suffering from PAOD (Lachmuth 2007) or CAD (Rothmann 2009). The demographic and clinical characteristics of the Caucasian patients and their medication prior to start of the study are summarized in Tables 1 and 2, respectively. These studies have been described in part elsewhere (Kayacelebi et al. 2015a, b). A detailed description of the studies has been reported elsewhere (Lachmuth 2007; Rothmann 2009). Inclusion criteria in the PAOD study were: Caucasian male and female patients older than 40 years with a peripheral arterial occlusive disease at Fontaine stage IIb and who had undergone cardiologic and angioplastic examination, with PAOD being the main limiting factor of the absolute walking distance. In the CAD study were included male and female patients aged 40–85 years who had undergone angioplasty. There were several exclusion criteria in both studies, which included heart failure with NYHA class III and IV, a creatinine clearance of ≤20 mL/min (according to Cockroft-Gault), hemodialysis, and organ transplantation.

PAOD and CAD patients received two $NaHCO_3$-based effervescent tablets (C. Hedenkamp GmbH, Hövelhof, Germany) that contained 2 g Arg hydrochloride (1.66 g Arg; Arginine group) or 2 g mannitol (Placebo group), thrice a day for 3 months in the PAOD study and for 6 months in the CAD study, resulting in a total daily amount of 9.96 g Arg in the Arginine groups in both studies. Mannitol is a widely and increasingly used pharmaceutical excipient in solid dosage forms (Ohrem et al. 2014). Orally administered mannitol is not systemically bioavailable. At the dose used in the placebo tablets, mannitol is unlikely to affect renal reabsorption of nitrite and nitrate. Yet, an effect on gut permeability is possible. The patients of the PAOD and CAD studies were not on special diets during the whole study periods. Yet, they have been asked to abstain from nitrite/nitrate-rich foods.

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in patients resting for 10 min in supine position on both arms after Riva-Rocci at the start, after 3 months in the PAOD and CAD studies, and after 6 months of the CAD study. PAOD patients underwent several tests including exercise test on treadmill. Absolute walking distance and pain-free walking distance and ankle-brachial index were measured in the PAOD study. Quality of life was assessed by the standardized questionnaire SF-12 (QualityMetric Inc., Lincoln, USA) Health Survey containing a mental (MCS) and a physical (PCS) component summary score.

Blood (9 mL EDTA monovettes) and urine samples from spontaneous micturition (40 mL) were collected

Table 1 Clinical and anthropometric characteristics of the PAOD patients of the Arginine and Placebo groups at baseline

	Arginine	Placebo	*P*
Number (*n*)	20	20	–
Gender (male/female)	16/4	15/5	1.000
Age (years)[a]	67.3 ± 8.0	68.4 ± 8.0	0.651
Body mass index (kg/m^2)[a]	27.4 ± 3.8	28.0 ± 4.4	0.700
Diabetes mellitus (%)	10	40	0.028
Arterial hypertension (%)	75	100	0.047
Hyperlipoproteinemia (%)	80	70	0.465
Coronary heart disease (%)	35	35	1.000
Heart insufficiency NYHA I–II (%)	0	5	1.000
Absolute walking distance (m)	122 ± 48	112 ± 54	0.389
Pain-free walking distance (m)	86 ± 39	82 ± 38	0.676
Systolic blood pressure (mmHg)	155 ± 21	168 ± 23	0.063
Diastolic blood pressure (mmHg)	81 ± 14	84 ± 11	0.369
Ankle-brachial-index (high-ABI)	0.55 ± 0.18	0.59 ± 0.28	0.831
Serum creatinine (mg/dL)	0.95 ± 0.26	0.98 ± 0.20	0.473
Creatinine clearance (mL/min)	90.5 ± 32.1	83.5 ± 34.4	0.511
Medication	*n* (%)	*n* (%)	*P*
Acetylsalicylic acid	17 (85)	14 (70)	0.451
Clopidogrel	1 (5)	4 (20)	0.342
Phenprocoumon	2 (10)	2 (10)	1.000
Pentoxifylline	0 (0)	1 (5)	1.000
Statins	17 (85)	10 (50)	0.018
Beta blockers	8 (40)	9 (45)	0.749
ACE inhibitors	12 (60)	12 (60)	1.000
Angiotensin II receptor antagonists	5 (25)	8 (40)	0.311
Calcium antagonists	5 (25)	5 (25)	1.000
Diuretics	9 (45)	14 (70)	0.110
Organic nitrates	2 (10)	3 (15)	1.000
Oral antidiabetics	1 (5)	4 (20)	0.342
Insulin	2 (10)	3 (15)	1.000

[a] Data are given as mean ± SD

in the morning after an overnight fasting at the start, after 3 months in the PAOD and CAD studies, and after 6 months in the CAD study. Blood was taken immediately before and immediately after the treadmill exercise, and urine was collected before the treadmill exercise in the PAOD study. Urine and plasma samples were stored immediately after collection at −20 °C until analysis.

The PAOD and CAD studies were approved by the Ethics Committee of the Hannover Medical School. Written informed consent was obtained from each patient.

Infusion of Arg in children with suspected GHD

Patients referred for suspected slow statural growth were included in the study and underwent the L-arginine test to diagnose GHD. This study has been described elsewhere in detail (Langen et al. 2015). The children were fasting overnight. Patients received infusion of an L-Arg·HCl solution in 190 mL 0.9 g % NaCl (0.5 g Arg/kg bodyweight) for 30 min. In a subgroup of 6 patients (2 children without GHD, 4 children with GHD) of the study (1 girl, 5 boys; aged, 9.9 ± 1.7 years; weight, 29.6 ± 9.5 kg), EDTA venous blood (2.7 mL) and urine from spontaneous micturition were collected immediately before and 90 min after the infusion was stopped. EDTA plasma and urine samples were stored at −80 °C and −20 °C until analysis.

The study was approved by the Ethics Committee of the Bochum University. Written informed consent was obtained from the parents.

Postprandial changes after the ingestion of high-fat protein meals in overweight men

Ten overweight (BMI > 25 kg/m^2; average BMI: 30.2 ± 1.5 kg/m^2, range 27.8–33.1 kg/m^2) men with enlarged waist circumference (>94 cm; average

Table 2 Clinical and anthropometric characteristics of the CAD patients of the Arginine and Placebo groups at baseline and medication

	Arginine	Placebo	*P*
Number (*n*)	31	29	–
Gender (male/female)	24/7	24/5	0.605
Age (years)[a]	62	62	0.905
Body mass index (kg/m^2)[a]	27	27	0.938
Diabetes mellitus (%)	29	31	0.866
Arterial hypertension (%)	77	66	0.307
Hypercholesterolemia (%)	84	69	0.173
PAOD (%)	13	10	1.000
Systolic blood pressure (mmHg)[b]	140	140	0.877
Diastolic blood pressure (mmHg)[b]	80	80	0.405
Medication	*n* (%)	*n* (%)	*P*
Platelet inhibitors	31 (100)	29 (100)	–
Statins	24 (77)	18 (62)	0.195
Beta®-Receptor blockers	23 (74)	24 (83)	0.421
ACE inhibitors	14 (45)	11 (38)	0.570
Angiotensin II receptor antagonists	8 (26)	5 (17)	0.421
Calcium antagonists	4 (13)	6 (21)	0.500
Diuretics	10 (32)	10 (35)	0.855
Organic nitrates	13 (42)	12 (41)	0.965
Oral antidiabetics	3 (10)	6 (21)	0.292
Insulin	1 (3)	2 (7)	0.606

[a] Data are given as mean or [b] median

96.3 ± 3.2 cm; range 94–103 cm), aged 21–50 years, without any serious illness, attended to 3 different day-long sessions at the research center, separated by at least 2 weeks. At each session, after an overnight fast, they ingested one of the three high-fat meals and were studied for 6 h thereafter. The meals had the same nutritional composition and protein content yet of different origin. The composition of the meals was as follows: energy, 1200 kcal; fat, 93.3 g (70 % energy); carbohydrates, 45 g (15 % energy); crude protein, 45 g (15 % energy). The test meals consisted of a mixture of 233 g 40 %-fat cream, 45 g sucrose, 45 g protein as protein isolates, and 160 mL water. The average amino acid content of the meals was as follows: arginine, 1.3 g; tryptophan, 1.0 g; threonine, 2.1 g; isoleucine, 2.3 g; leucine, 4.7 g; lysine, 4.0 g; methionine, 1.1 g; cysteine, 1.4 g; phenylalanine, 1.8 g; tyrosine, 1.9 g; valine, 2.5 g; histidine, 1.1 g; alanine, 1.6 g; aspartic acid, 4.6 g; glutamic acid, 7.9 g; glycine, 0.9 g; proline, 2.7 g; serine, 2.1 g. Urine from spontaneous micturition was collected before the meal (time zero, T0) and 2 h (T2), 4 h (T4) and 6 h (T6) after the meal. This study has been described elsewhere in detail (Kayacelebi et al. 2015a, b). The study was approved by the Institutional Review Board for Saint-Germain-en-Laye Hospital and authorized by the French Ministry of Health.

Biochemical analyses

With exception of cGMP, all biochemical parameters, i.e., nitrite, nitrate, Arg, ADMA, dimethylamine (DMA), which is the major urinary metabolite of ADMA (Achan et al. 2003), the major urinary metabolites of prostacyclin (PGI_2) and thromboxane (TxA_2), 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ and 2,3-dinor-thromboxane B_2, respectively (Tsikas 1998), and creatinine were analyzed in plasma and/or urine samples by previously reported fully validated mass spectrometry-based methods (Tsikas 2000, 2008; Tsikas et al. 2000a, b, 2003, 2014). cGMP was determined in urine samples by means of a commercially available ELISA kit from R&D systems GmbH (Wiesbaden-Nordenstadt, Germany). The urinary excretion of the analytes was corrected for creatinine excretion and is expressed in pmol, nmol or µmol analyte per mmol creatinine.

Statistical analyses

Statistical analyses were performed by means of the Software SPSS 14.0 for Windows. Graphs were constructed by GraphPad Prism 5.04 (GraphPad Prism Software Inc. San Diego, California, USA). Distribution of variables was tested by Kolmogorov–Smirnov or D'Agostino and Pearson omnibus K2 test. Normally distributed parameters were compared by parametric tests (Student's *t* test) and are presented as mean ± standard

Table 3 Biochemical parameters measured in the plasma samples of the PAOD patients study in the Arginine group ($n = 20$) and Placebo group ($n = 20$) before and after 3 months of supplementation with Arg or placebo

Biochemical parameter[a]	Arginine group	Placebo group	*P*
Arg baseline	56.5 ± 19.0 (55.4)	56.4 ± 15.5 (58.2)	0.992
Arg after	80.0 ± 37.8 (71.1)*	50.9 ± 17.3 (46.9)	0.002
ADMA baseline	0.51 ± 0.14 (0.51)	0.59 ± 0.21 (0.54)	0.152
ADMA after	0.55 ± 0.10 (0.54)	0.54 ± 0.11 (0.51)	0.792
Arg/ADMA baseline	112 ± 36.8 (109)	99.0 ± 26.3 (102)	0.191
Arg/ADMA after	146 ± 59.8 (132)*	95.0 ± 31.3 (94.8)	0.002
Nitrate baseline PreEx	40.8 ± 13.8 (38.7)	40.7 ± 13.8 (37.4)	0.914
Nitrate after PreEx	45.4 ± 21.4 (37.0)	41.0 ± 14.7 (40.5)	0.808
Nitrate baseline PostEx	41.7 ± 13.2 (38.8)	41.2 ± 13.2 (38.0)	0.935
Nitrate after PostEx	46.2 ± 22.2 (37.7)	40.6 ± 14.2 (38.5)	0.685
Nitrite baseline PreEx	1.37 ± 0.50 (1.37)	1.18 ± 0.43 (1.18)	0.123
Nitrite after PreEx	1.28 ± 0.55 (1.08)	1.20 ± 0.47 (1.14)	0.695
Nitrite baseline PostEx	1.31 ± 0.48 (1.26)	1.38 ± 0.56 (1.32)	0.661
Nitrite after PostEx	1.38 ± 0.60 (1.33)	1.41 ± 0.48 (1.34)	0.856
$P_{NOX}R$ baseline PreEx	33.1 ± 14.1 (46.2)	37.8 ± 15.3 (47.2)	0.417
$P_{NOX}R$ after PreEx	39.6 ± 18.9 (54.5)	37.6 ± 15.7 (34.4)	0.787
$P_{NOX}R$ baseline PostEx	34.9 ± 12.6 (45.9)	33.0 ± 12.2 (31.3)	0.636
$P_{NOX}R$ after PostEx	37.4 ± 16.5 (50.8)	30.4 ± 11.0 (27.1)	0.190

Asterisk statistical significance within the same group when compared baseline with 3 months

PreEx pre-exercise, *PostEx* post-exercise

[a] Data (in μM) are reported as mean ± SD (median) for $n = 20$ for each group

deviation (SD) or standard error of the mean (SEM). Non-normally distributed parameters were analyzed by non-parametric tests (Mann–Whitney test) and are presented as median and interquartile range (25th–75th percentile) or 95 % confidence interval. Correlations between variables were assessed by Pearson (parametric) or Spearman (non-parametric) correlation. *P* values <0.05 were considered as statistically significant.

Results

Effects of chronic oral L-arginine administration on the Arg/NO pathway and $U_{NOX}R$ in elderly PAOD and CAD patients

Arginine was well tolerated by the PAOD and CAD patients. There were no statistically significant differences with respect to side effects between the Arginine and Placebo groups in both studies. Diarrhea occurred in 3 patients (shorter than 3 days) of the Arginine group and in 5 patients (3 patients longer than 3 days) of the Placebo group ($P = 0.695$) of the PAOD study. In the CAD study, diarrhea (shorter than 3 days) occurred in 5 patients of the Arginine group and in 2 patients (3 patients longer than 3 days) of the Placebo group ($P = 0.450$).

In addition to the biochemical parameters which are discussed below for each study separately, we measured in the studies several clinically relevant parameters including absolute walking distance in the PAOD study, as well as blood pressure and quality of life in both studies. All these parameters did not change significantly in both groups after the 3- or 6-month treatment.

PAOD study

Forty-two patients were included in the study. The patients were recruited and examined by the Department of Cardiology at the Hannover Medical School (Hannover, Germany). One patient from the Arginine group and one patient of the Placebo group discontinued the study; the corresponding results were not considered in the statistical analysis. The results of the PAOD study are summarized in Table 3 for the biochemical parameters in plasma and in Table 4 for those in urine including the $U_{NOx}R$.

Expectedly, Arg supplementation increased both the Arg plasma concentration (80 vs 57 μM, $P < 0.05$) and the urinary excretion of Arg (4.56 vs 3.14 μmol/mmol creatinine, $P < 0.05$) by about 30 % each. The urinary excretion of Arg correlated positively with the plasma concentration of Arg in the Arginine group ($r = 0.382$, $P = 0.015$) but not in the Placebo group ($r = 0.218$, $P = 0.176$) (Fig. 2).

Oral intake of Arg increased insignificantly the ADMA concentration in the plasma but enhanced the excretion rate of ADMA. As the excretion of DMA, the major urinary metabolite of ADMA, did not change upon Arg intake (59 vs 44 μmol/mmol creatinine, $P > 0.05$), the net effect of Arg administration on ADMA synthesis is not clear. Yet, Arg administration seems to have elevated whole body ADMA synthesis. The increase in plasma Arg was more pronounced than that of ADMA. Therefore, Arg supplementation increased the Arg/ADMA molar ratio in the Arginine group (146:1 vs 112:1, $P < 0.05$). However, this increase did not result in appreciable increase in systemic and whole body NO synthesis and bioavailability. This is indicated by the lack of changes in plasma and urine concentrations of nitrate (45.4 vs 41.7 μM, $P > 0.05$; 85.7 vs 79.7 μmol/mmol creatinine, $P > 0.05$), nitrite (1.28 vs 1.37 μM, $P > 0.05$; 230 vs 277 nmol/mmol creatinine, $P > 0.05$), and urinary cGMP (58.1 vs 54.2 nmol/mmol creatinine, $P > 0.05$).

Table 4 Biochemical parameters measured in the urine samples of the PAOD study in the Arginine and Placebo groups before and after 3 months of supplementation with Arg or placebo

Analyte	Arginine	Placebo	*P*
Arg baseline	3.14 ± 1.39 (2.76)	3.19 ± 1.01 (2.72)	0.692
Arg after	4.56 ± 1.57 (4.73)*	3.55 ± 1.32 (3.25)	0.034
ADMA baseline	3.02 ± 0.83 (3.11)	2.89 ± 0.73 (2.87)	0.433
ADMA after	3.37 ± 0.68 (3.15)*	3.13 ± 0.89 (2.99)	0.342
DMA baseline	59.2 ± 102 (36.2)	69.3 ± 97.7 (37.1)	0.808
DMA after	44.0 ± 20.5 (39.0)	35.6 ± 9.7 (34.4)*	0.128
DMA/ADMA baseline	20.0 ± 29.0 (12.9)	24.5 ± 33.2 (13.3)	0.402
DMA/ADMA after	13.4 ± 6.0 (11.4)	12.2 ± 4.5 (11.7)*	0.550
cGMP baseline	54.2 ± 18.5 (55.9)	60.5 ± 17.9 (63.6)	0.283
cGMP after	58.1 ± 21.3 (57.4)	64.9 ± 23.0 (64.4)	0.336
Nitrate baseline	79.7 ± 41.2 (65.5)	85.1 ± 45.4 (78.3)	0.372
Nitrate after	85.8 ± 36.6 (80.0)	84.6 ± 27.2 (78.0)	0.904
Nitrite baseline	277 ± 363 (143)	287 ± 264 (214)	0.279
Nitrite after	230 ± 171 (149)	323 ± 159 (309)	0.006
$U_{NOx}R$ baseline	480 ± 51 (461)	422 ± 67 (342)	0.049
$U_{NOx}R$ after	486 ± 50 (545)	332 ± 42 (316)*	0.026
PGI_2 baseline	26.8 ± 11.4 (26.6)	33.7 ± 24.1 (25.3)	0.603
PGI_2 after	26.0 ± 15.8 (25.3)	42.0 ± 26.9 (35.5)*	0.025
TxA_2 baseline	50.1 ± 45.1 (37.7)	59.4 ± 53.8 (45.2)	0.772
TxA_2 after	44.4 ± 45.9 (28.1)	57.2 ± 54.2 (23.3)	0.766
PGI_2/TxA_2 baseline	0.82 ± 0.57 (0.67)	1.10 ± 1.69 (0.61)	0.936
PGI_2/TxA_2 after	0.90 ± 0.73 (0.68)	1.32 ± 1.08 (0.89)	0.182

Asterisk statistical significance within the same group when compared baseline with 3 months

Arg, ADMA, DMA, nitrate: μmol/mmol creatinine; Nitrite, cGMP: nmol/mmol creatinine; PGI_2, TxA_2: pmol/mmol creatinine

[a] Data are given as mean ± SD (median)

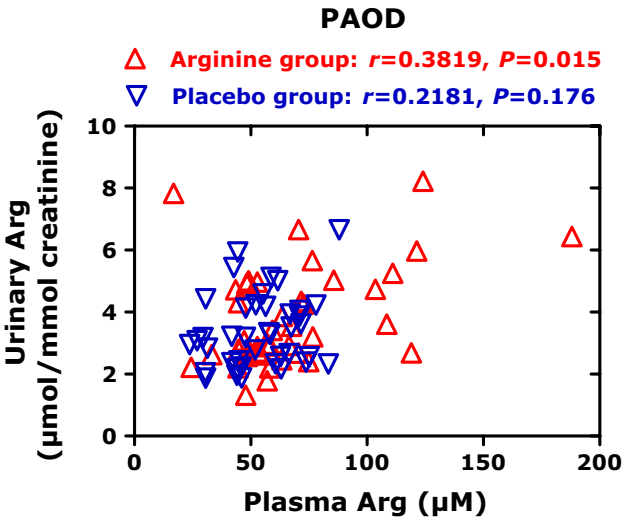

Fig. 2 Relationship between urinary Arg excretion and plasma Arg concentration in the Arginine and Placebo groups of the PAOD study at baseline and after 3 months

Baseline serum creatinine and creatinine clearance (according to Cockcroft-Gault) did not differ between the groups (Table 1). Serum creatinine did not differ between the Arginine and Placebo groups at baseline [0.93 (0.76–1.04) vs 0.97 (0.84–1.02) mg/dL, $P = 0.482$] and at the end of the study [0.85 (0.74–0.99) vs 0.90 (0.82–0.95) mg/dL, $P = 0.636$]. Neither Arginine ($P = 0.534$) nor Placebo ($P = 0.232$) had an effect on serum creatinine concentration. Urinary creatinine did not differ between the Arginine and Placebo groups at baseline [9.3 (5.3–13.6) vs 6.3 (4.1–9.4) mM, $P = 0.126$], but it did differ at the end of the study [9.1 (6.1–11.8) vs 6.7 (3.6–8.0) mM, $P = 0.027$]. Neither Arginine ($P = 0.715$) nor Placebo ($P = 0.422$) had an effect on urinary creatinine concentration after 30 weeks of treatment.

The nitrate-to-nitrite molar ratio in urine, $U_{NOx}R$, may be an appropriate measure of the relative loss of NO bioactivity due to a differential excretion of nitrate and nitrite in the urine. As the urinary excretion of nitrite depends upon the activity of renal CA isoforms (Fig. 1), the $U_{NOx}R$ value may serve as a measure of the nitrite-dependent CA activity. The mean $U_{NOx}R$ value determined in the 40 PAOD patients of the present study at baseline (480:1 in the Arginine group; 422:1 in the Placebo group) is lower compared to the mean $U_{NOx}R$ value of 626:1 in 30 non-medicated elderly healthy adults (Tsikas et al. 2015). This observation may indicate impaired reabsorption of nitrite in the investigated PAOD patients due to altered nitrite-dependent CA activity in the kidney. Although not statistically significant, the combination of apparently slightly enhanced synthesis of NO and slightly decreased excretion of nitrite resulted in constant $U_{NOx}R$ values in the Arginine group (486:1 vs 480:1, $P > 0.05$). In contrast, in the Placebo group NO synthesis was not elevated after 3 months. Moreover, the increase in nitrite excretion after 3 months supplementation with placebo resulted in a remarkable decrease of the $U_{NOx}R$ value from 422:1 to 332:1 ($P < 0.05$). That $U_{NOx}R$ did not change in the Arginine group but did decrease in the Placebo group, is likely to be due to the Arg supplementation. Neither plasma nor urinary Arg correlated with $U_{NOx}R$ in both groups at baseline and after 3 months (data not shown). In the Placebo group, 8 PAOD patients also suffered from diabetes (Table 1). The $U_{NOx}R$ value did not differ between diabetic and non-diabetic PAOD patients of the Placebo group at baseline (378 ± 71 vs 451 ± 104, $P = 0.38$) or after 3 months (324 ± 63 vs 338 ± 59, $P = 0.67$). The nitrate-to-nitrite molar ratio in plasma, $P_{NOx}R$, did not differ between the groups at baseline and after 3 months in the PAOD study and did not change upon treatment with Arginine of Placebo (Table 3). Finally, the

decadic logarithm of the product of $U_{NOx}R$ and $P_{NOx}R$ [$\log_{10}(U_{NOx}R \times P_{NOx}R)$], which considers nitrate and nitrite changes in urine and plasma, did not change upon treatment in the Arginine [4.13 (3.96–4.31) vs 4.33 (3.90–4.39)] and Placebo [4.16 (3.82–4.32) vs 3.97 (3.68–4.26)] groups. Comparing Arginine and Placebo group revealed a difference for $\log_{10}[U_{NOx}R \times P_{NOx}R]$ at the end ($P = 0.03$) but not at the start ($P = 0.63$). This finding supports a loss of nitrite in the Placebo but not in the Arginine group of the PAOD study.

The Arg/NO pathway is closely related to the arachidonic acid pathway (Ahmetaj-Shala et al. 2015). The latter generates two antagonistically acting lipid mediators, i.e., prostacyclin (PGI_2) and thromboxane A_2 (TxA_2): PGI_2 is a potent vasodilator and inhibitor of platelet aggregation, whereas TxA_2 is a vasoconstrictor and inductor of platelet aggregation (Fitzgerald and Fitzgerald 2013). In the PAOD study, Arg supplementation did not change significantly the homeostasis of PGI_2 and TxA_2. In the Placebo group, systemic PGI_2 synthesis increased significantly after 3 months (42 vs 34 pmol/mmol creatinine, $P < 0.05$). While the Arginine and Placebo groups did not differ significantly at baseline regarding systemic PGI_2 synthesis (27 vs 34 pmol/mmol creatinine, $P = 0.603$), the difference was significant after 3 months of supplementation with Arginine or Placebo (26 vs 42 pmol/mmol creatinine, $P = 0.025$). Yet, this change was not associated with an alteration of the vasodilation/vasoconstriction balance which is expressed by the PGI_2/TxA_2 molar ratio (i.e., 1.3:1 vs 0.9:1, $P = 0.182$).

In the Arginine group, there was no correlation between the DMA/ADMA and PGI_2/TxA_2 ratios ($r = -0.29$, $P = 0.24$ at baseline vs $r = -0.34$, $P = 0.15$ after 3 months; Fig. 3a, b). In the Placebo group, there was a close negative correlation between the DMA/ADMA and PGI_2/TxA_2 ratios at baseline, which decreased slightly after 3 months ($r = -0.72$, $P = 0.001$ vs $r = -0.45$, $P = 0.053$; Fig. 3c, d).

In the PAOD study, the average SBP/DBP was 155/81 vs 153/76 mmHg in the Arginine group and 168/84 vs 166/81 mmHg in the Placebo group at baseline and after 3 months, respectively. A statistically significant difference between the Arginine group and the Placebo group was observed for DBP (76 vs 81 mmHg, $P = 0.038$). In the Arginine group, DBP ($r = -0.55$, $P = 0.01$) but not SBP ($r = -0.20$, $P = 0.39$) correlated with $U_{NOx}R$ at baseline (Fig. 4a). Yet, after 3 months of Arg supplementation, the correlation between DBP and $U_{NOx}R$ was not significant (Fig. 4b).

CAD study

The patients of this study were recruited and examined by two study centers in Oldenburg and Magdeburg (both Germany). In total, 364 patients were examined and 134 patients were enrolled in the study. In total, 74 patients discontinued the study. The results of the CAD study are summarized in Table 5 for the biochemical parameters in plasma and in Table 6 for those in urine, including the $U_{NOx}R$.

Compared to Placebo, Arg supplementation to CAD patients for 6 months increased the Arg plasma concentration (94.7 vs 60.6 µM, $P < 0.05$), whereas plasma ADMA, nitrite and nitrate did not change significantly. Compared to baseline, Arg excretion in the urine increased after 3 and 6 months, with the increase after 3 months being more pronounced (2.76 vs 5.10 vs 3.55 µmol/mmol creatinine) in the Arginine group. Urinary ADMA increased only marginally after 3 but not after 6 months of Arg supplementation (4.10 vs 4.29 vs 4.24 µmol/mmol creatinine). Urinary excretion of DMA, nitrate and nitrite did not significantly change after Arg supplementation for 3 and 6 months. At baseline, the CAD patients in the Arginine and Placebo groups had almost identical $U_{NOx}R$ values (518 ± 77 vs 525 ± 69, $P = 0.828$), which decreased after treatment with Arginine or Placebo. The $U_{NOx}R$ fall was less pronounced in the Arginine group after 3 and 6 months. The $U_{NOx}R$ values determined in the 60 CAD patients of the present study at baseline are very close to those measured in the PAOD patients and lower compared to that of elderly healthy adults (Tsikas et al. 2015). This observation may indicate impaired reabsorption of nitrite in the investigated CAD patients, too. Neither plasma nor urinary Arg correlated with $U_{NOx}R$ in both groups of the CAD study at baseline and after 3 and 6 months (data not shown).

Effect of infused L-arginine on $U_{NOX}R$ in children

Intravenous infusion of large amounts of Arg over 30 min is routinely used clinically to test growth hormone deficiency (GHD) in children. Arginine was well tolerated by the children who underwent the L-arginine test (Langen et al. 2015). In 6 children who underwent the L-arginine test, we measured the urinary excretion of nitrate, nitrite and creatinine in urine and the plasma concentration of Arg. Arg infusion did not reduce significantly the creatinine concentration in the urine [5.27 (3.22–11.8) mM vs 3.04 (2.66–5.43) mM, $P = 0.109$; Wilcoxon matched-pairs rank test]. Immediately before starting the Arg infusion, $U_{NOx}R$ and plasma Arg were determined to be (mean ± SEM) 317 ± 41 and 81 ± 7 µM, respectively. Two hours after starting the Arg infusion, $U_{NOx}R$ and plasma Arg were determined to be 208 ± 16 and 459 ± 62 µM, respectively (Fig. 5). The $U_{NOx}R$ fell by about 30 % but the decrease barely failed statistical significance ($P = 0.062$), whereas plasma Arg concentration was more than five times higher compared to the baseline value ($P = 0.002$). The $U_{NOx}R$ fall

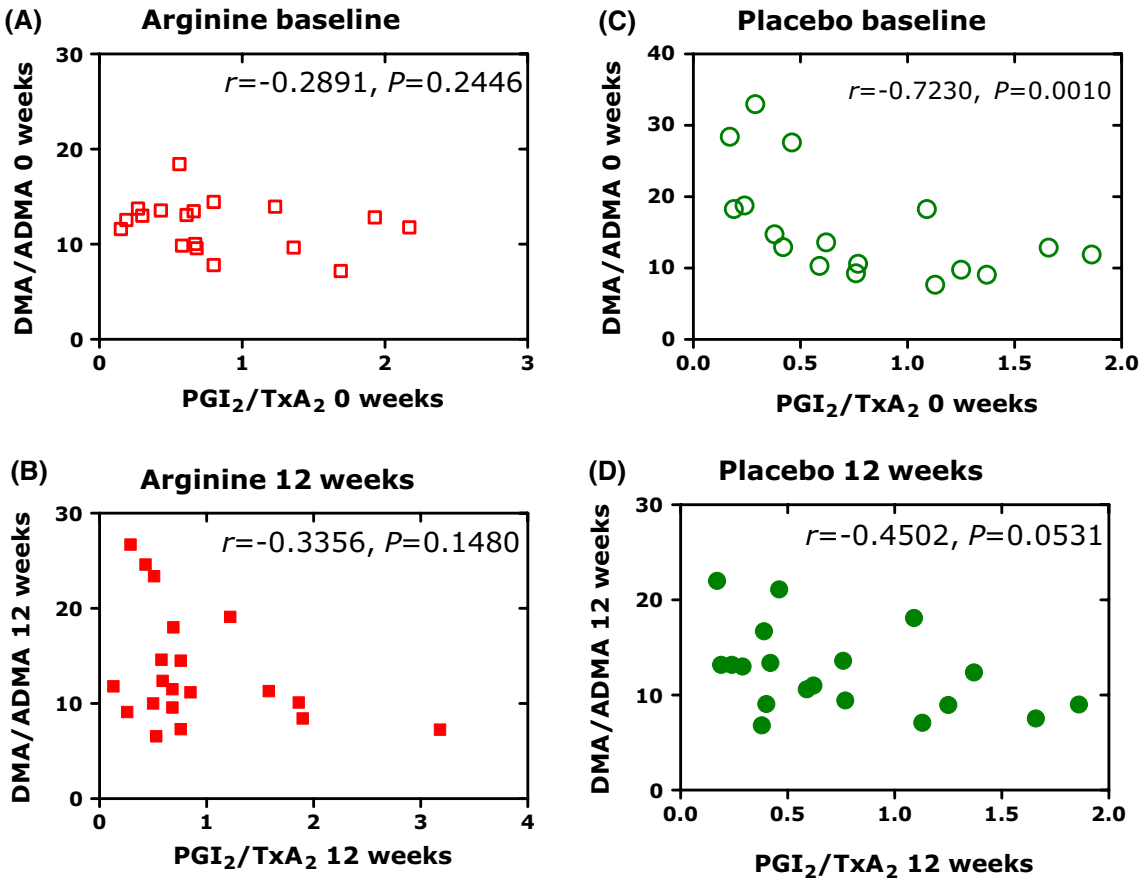

Fig. 3 Relationship between the urinary DMA/ADMA ratio and the PGI_2/TxA_2 ratio in the Arginine and Placebo groups of the PAOD study at baseline and after 3 months (12 weeks)

was mainly caused by an enhanced excretion of nitrite in the urine (0.7 vs 0.5 µmol/mmol creatinine), without appreciable changes in nitrate excretion (149 vs 137 µmol/mmol creatinine). The average maximum plasma Arg concentration in the children was about 1440 µM immediately after stopping the Arg infusion. These results suggest that intravenous infusion of high amounts of Arg results in very high plasma concentrations of Arg which are associated with a remarkable decrease (by about 35 %) in the $U_{NOx}R$ value 90 min after completed infusion. This may be explained by the enhanced urinary nitrite excretion, presumably due to altered nitrite-dependent renal CA activity.

Effect of high-fat protein meals on $U_{NOX}R$ in overweight men

The baseline $U_{NOx}R$ value was 369 ± 33 in the ten overweight but otherwise healthy men. Ingestion of high-fat protein meals did not change the $U_{NOx}R$ value 2, 4 or 6 h after the meals (Fig. 6). The corresponding plasma Arg concentrations were (mean ± SEM) 85.2 ± 2.5, 93.6 ± 3.3, 82.8 ± 2.9 and 70.8 ± 2.8 µM.

Discussion

The semi-essential proteinogenic amino acid L-arginine (Arg) is the substrate of the endothelial NOS (eNOS). Arachidonic acid/cyclooxygenase (COX)-derived prostacyclin (PGI_2) and Arg/eNOS-derived NO produced in endothelial cells are potent vasodilators and inhibitors of platelet aggregation in the vasculature. In healthy humans, only a very low proportion of arachidonic acid and Arg (presumably less than 0.1 % Arg) is utilized for the COX- and eNOS-catalyzed synthesis of PGI_2 and NO, respectively, underlining the high potency of PGI_2 and NO in the circulation.

The baseline Arg concentration measured in plasma of all studies presented here are within the reference intervals for plasma Arg in a general population (Lüneburg et al. 2011). Despite saturation of eNOS by its substrate Arg (the K_M value is in the lower µM range for all NOS isoforms), oral and intravenous supplementation of Arg may enhance NO synthesis (Bode-Böger 2006; Mariotti et al. 2013), a phenomenon widely known as "The L-arginine paradox" (Tsikas et al. 2000a, b; Böger 2004). Yet, Arg is not only a source of NO, but is also a source for guanidine-methylated

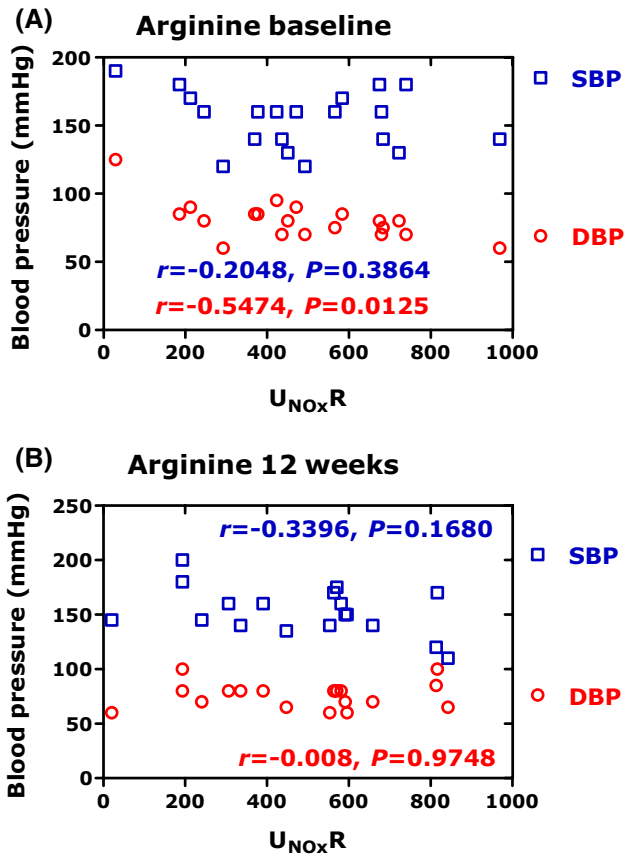

Fig. 4 Relationship between the systolic blood pressure (SBP) or diastolic blood pressure (DBP) and the urinary nitrate-to-nitrite molar ratio ($U_{NOx}R$) in the Arginine group of the PAOD study at baseline and after 3 months (12 weeks)

Table 5 Biochemical parameters (in μM) measured in the plasma samples of the CAD patients study in the Arginine group ($n = 31$) and Placebo group ($n = 29$) at baseline and after 6 months of supplementation

Biochemical parameter[a]	Arginine group	Placebo group	*P*
Arg baseline	67.7 (58.5–73.5)	58.9 (52.1–64.4)	0.108
Arg after 6 months	94.7 (87.4–112)*	60.6 (52.9–66.4)	<0.001
ADMA baseline	0.50 (0.47–0.52)	0.46 (0.45–0.50)	0.374
ADMA after 6 months	0.50 (0.46–0.53)	0.47 (0.44–0.62)	0.544
Arg/ADMA baseline	138 (96–151)	125 (106–148)	0.680
Arg/ADMA after 6 months	189 (151–226)*	123 (94–149)	<0.001
Nitrate baseline	41.0 (38.5–52.3)	42.8 (35.4–74.8)	0.717
Nitrate after 6 months	35.6 (34.5–45.3)	35.6 (35.4–47.5)*	0.979
Nitrite baseline	1.55 (1.32–1.65)	1.60 (1.36–1.93)	0.615
Nitrite after 6 months	1.51 (1.27–1.84)	1.43 (1.26–1.55)	0.669

Asterisk statistical significance within the same group when compared baseline with 6 months

[a] Data are reported as median 95 % CI

(N^G) arginine analogs MMA, ADMA and SDMA, which are all inhibitors of eNOS activity at concentrations lying in the low μM range (Tsikas et al. 2000a, b). Thus, the IC_{50} of ADMA towards recombinant eNOS is 12 μM, but 1.5 μM towards recombinant nNOS (Kielstein et al. 2007). Concomitant synthesis of MMA, ADMA and SDMA from supplemented Arg, presumably after its incorporation in proteins, and other not yet identified phenomena that follow the increase in Arg concentrations in the body, may outweigh and even outbalance the promoting effects of Arg on NO synthesis. The PAOD and CAD patients supplemented with Arg had higher plasma Arg concentrations and excreted higher amounts both of ADMA and Arg compared to baseline. Therefore, Arg supplementation may not mandatorily result in enhanced NO synthesis/bioavailability and increased NO-related actions such as blood pressure lowering. Yet, in addition to serve as a substrate for NOS, Arg supplementation may enhance NO synthesis in endothelial cells by increasing the synthesis of tetrahydrobiopterin, an essential cofactor for all isoforms of NOS (Shi et al. 2004). As circulating and excretory nitrite and nitrate do not reflect specifically the activity of a certain NOS isoform (Tsikas et al. 2015) and because the activity of eNOS is very low compared to nNOS and iNOS (Böhmer et al. 2014), lack in increases in nitrite and nitrate concentrations in blood and/or urine may not necessarily indicate absence of Arg effects on NO synthesis and bioavailability in particular cells such as endothelial cells.

In previous study, we found that performing the L-arginine test in ten healthy male subjects, i.e., intravenous infusion of Arg (30 g in 30 min), resulted in a significantly lowered blood pressure and in concomitantly increased plasma nitrite and cGMP concentrations, with these effects being most likely mediated by Arg-induced elevation in growth hormone (Bode-Böger et al. 1994, 1999). In the PAOD study, Arg supplementation decreased significantly the DBP (76 vs 81 mmHg, $P = 0.038$), which may have resulted from a higher concentration of nitrite in the circulation. A decrease in DBP was also observed during the L-arginine test in healthy adults (Bode-Böger et al. 1994) as well as in patients with PAOD of a previous study, who received 30 g of Arg intravenously (Bode-Böger et al. 1996). In previous study, we also found a decrease in DBP after oral supplementation of 9 g Arg per day in hypercholesterolemic men (West et al. 2005). At present, we have no explanation for the disappearance of the correlation between DBP and $U_{NOx}R$ that existed at baseline. Intravenous Arg infusion in eight male subjects induced hypotension and diuresis/natriuresis with concomitantly increased urinary excretion of the sum of nitrate and nitrite (Kanno et al. 1992). Similar results were also observed during the L-arginine test in 6 cirrhotic patients with ascites and 5 healthy subjects (Tajiri et al. 1995). Interestingly,

Table 6 Biochemical parameters (in µmol/mmol creatinine) measured in the urine samples of the CAD study in the Arginine and Placebo groups at baseline, after 3 and 6 months of supplementation

Analyte	Arginine	Placebo	*P*
Arg baseline	2.76 (2.39–3.33)	2.38 (2.23–3.07)	0.518
Arg after 3 months	5.10 (4.47–6.69)*	2.59 (2.45–3.71)	<0.001
Arg after 6 months	3.55 (3.67–6.05)*	2.71 (2.40–3.51)	0.004
ADMA baseline	4.10 (3.73–4.85)	3.58 (3.52–4.53)	0.299
ADMA after 3 months	4.29 (4.07–5.10)*	3.77 (3.48–4.26)	0.040
ADMA after 6 months	4.24 (3.81–5.04)	0.67 (3.31–4.30)	0.123
DMA baseline	35.7 (36.0–51.0)	35.7 (35.0–42.6)	0.798
DMA 3 months	37.0 (33.8–47.9)	34.4 (31.6–39.6)	0.198
DMA 6 months	36.9 (28.4–59.2)	35.4 (33.1–46.7)	0.972
Nitrate baseline	78.9 (71.7–117)	87.0 (79.6–125)	0.544
Nitrate 3 months	92.0 (83.5–126)	69 (64.7–89.9)*	0.025
Nitrate 6 months	73.0 (64.3–88.2)*	61.0 (50.7–71.0)*	0.036
Nitrite baseline	0.20 (0.16–0.57)	0.19 (0.13–0.58)	0.883
Nitrite 3 months	0.27 (0.22–0.40)	0.26 (0.28–0.61)*	0.549
Nitrite 6 months	0.21 (0.19–0.51)	0.25 (0.21–0.37)	0.618
$U_{NOx}R$ baseline	518 ± 77 (406)	525 ± 69 (409)	0.828
$U_{NOx}R$ 3 months	422 ± 40 (430)	302 ± 36 (273)*	0.029
$U_{NOx}R$ 6 months	399 ± 66 (300)	285 ± 31 (273)*	0.297

Asterisk statistical significance within the same group when compared baseline with 3 or 6 months

[a] Data are given as median 95 % CI, except for $U_{NOx}R$ [mean ± SD (median)]

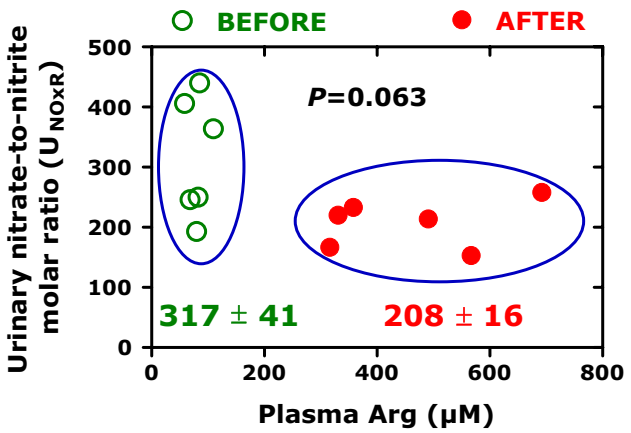

Fig. 5 Relationship between the urinary nitrate-to-nitrite molar ratio ($U_{NOx}R$) immediately before starting (BEFORE) and 90 min after (AFTER) stopping the Arg infusion in six children of the GHD study

intravenous Arg infusion in that study lowered DBP but not SBP in the healthy subjects. Yet, the individual urinary nitrate and nitrite concentrations have not been reported in those studies (Kanno et al. 1992; Tajiri et al. 1995), so that the $U_{NOx}R$ values cannot be calculated.

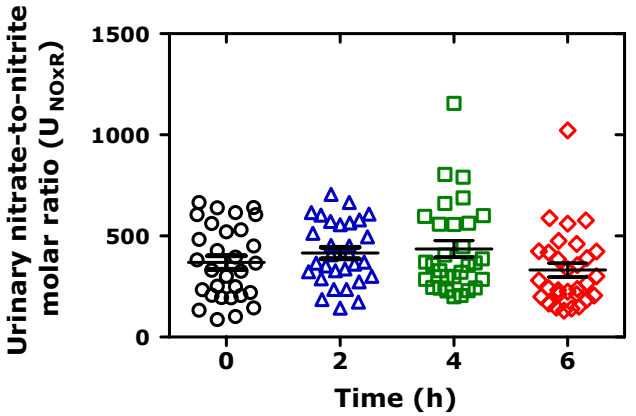

Fig. 6 Urinary nitrate-to-nitrite molar ratio ($U_{NOx}R$) before (0 h) and 2 h, 4 h and 6 h after high-fat meals ingestion by ten overweight men. Data are shown as mean ± SEM

Besides the liver, the kidney plays an important role in biosynthesis, metabolism, elimination and reabsorption of Arg, SDMA, ADMA, and their metabolites, including the ADMA metabolite DMA and the NO metabolites nitrite and nitrate. In humans, the kidney produces about 2 g Arg/day, which may be compared to an intake, from a Western diet, of approximately 4 to 5 g/day (Brosnan and Brosnan 2004). Arg is reabsorbed from the primary urine in the proximal tubule of the nephron. Reabsorption of Arg in the kidney salvages about 3 g Arg/day (Brosnan and Brosnan 2004). Especially nitrite reabsorption in the kidney is dependent upon the activity of renal carbonic anhydrase (CA) isoforms, because the CA inhibitor acetazolamide inhibits reabsorption both of endogenous and of exogenous nitrite (Tsikas et al. 2010a, b; Chobanyan-Jürgens et al. 2012). In the Arginine groups of our PAOD and CAD studies, the $U_{NOx}R$ value did not decrease (PAOD) or did decrease less strongly when compared with the Placebo groups. It is, therefore, possible that intake of Arg of about 10 g/day for 3 months by the PAOD and CAD patients did improve the nitrite-related activity of renal CA isoforms and/or did prevent a stronger loss of nitrite in the urine by maintaining the reabsorption efficiency in the kidney. In our PAOD and CAD studies, the main effect of long-term Arg supplementation of 10 g/day, which is twice the normal intake rate of Arg by nutrition in Western countries (Brosnan and Brosnan 2004), seems to be saving nitrite which in turn bears NO bioactivity, rather than enhancing NOS-catalyzed synthesis of NO from Arg. Interestingly, based on the PGI_2/TxA_2 molar ratio assessed by measuring their major urinary metabolites (Tsikas 1998), long-term Arg supplementation did not alter the vasodilation/vasoconstriction balance in the PAOD patients.

The $U_{NOx}R$-decreasing effect of infused Arg in the children suggests that high Arg concentrations in the blood, as

they resulted from infusion of high amounts of Arg in the children, may reduce nitrite reabsorption from the urine. It can, therefore, not be excluded that even lower Arg concentrations, as a result of oral intake of Arg, may also have diminished nitrite reabsorption to a higher extent in the CAD study in which patients received the same Arg dose but for a twice as long period of time. In many studies, Arg has been supplemented in high amounts to enhance the NO synthesis. Yet, our studies suggest that elevation of NO synthesis by supplementing high amounts of Arg may be prevented by inhibiting nitrite reabsorption. The optimum Arg supplementation dose in PAOD, CAD and other diseases associated with NO-related dysfunction remains to be established. Ingestion of high-fat meal by overweight men did not alter the $U_{NOx}R$ value. Whether the remarkably postprandial constancy of the $U_{NOx}R$ value is due to the average increase of 9 % in plasma Arg concentration or due to other constituents of the meals is unknown.

A further potentially important aspect that needs to be addressed here is that Arg was supplemented in both studies in the form of effervescent tablets which contained $NaHCO_3$. As in the Arginine and Placebo groups of both studies no changes were seen in serum Na^+ and HCO_3^- concentrations (data not reported) and in the placebos the same amount of $NaHCO_3$ was present, $NaHCO_3$ is unlikely to have influenced nitrite excretion/reabsorption in the PAOD and CAD studies. Further, the patients of the PAOD and CAD studies suffered from additional diseases such as diabetes and received different medications such as organic nitrates which also contribute to nitrite and nitrate in plasma and urine (Keimer et al. 2003). These factors may have influenced the reabsorption of nitrite and consequently the $U_{NOx}R$ value. The number of PAOD patients who were diabetic was relatively small and did not allow statistical analysis. That the $U_{NOx}R$ value did not differ between diabetic and non-diabetic PAOD patients of the Placebo group both at baseline ($P = 0.38$) and after 3 months ($P = 0.67$), may suggest that diabetes did not further reduce the reabsorption of urinary nitrite. In the PAOD study, there were some patients on statins. However, the $U_{NOx}R$ value did not differ between the PAOD patients who received statins compared to those who did not. Statins seem not to influence the reabsorption of urinary nitrite in PAOD. Previously, we demonstrated that elderly subjects with type 2 diabetes mellitus have very low $U_{NOx}R$ values compared to healthy elderly subjects. Atorvastatin administration both at the standard dose of 10 mg/d and at the maximal dose of 80 mg/day for 30 weeks did not change $U_{NOx}R$ (Tsikas et al. 2015). Eventually, mannitol was used in the Placebo but not in the Arginine tablets. Whether orally administered mannitol affects reabsorption of nitrite and/or nitrate in the kidney is unknown and is unlikely because mannitol does not reach the circulation. Although mannitol may influence nitrite and nitrate absorption in gut, the nitrite and nitrate concentrations measured in the plasma of the PAOD and CAD patients did not differ between the Arginine and Placebo groups.

In summary, oral Arg supplementation to the study patients who suffered from PAOD or CAD of 10 g/day for 3 or 6 months was as well tolerated as the patients who received the placebo effervescent tablets. At this dosage, Arg did not influence the quality of life in both studies, did not prolong the walking distance in the PAOD study, and did not alter the synthesis/bioavailability of NO (in PAOD and CAD), PGI_2 and TxA_2 (in PAOD). However, Arg supplementation for 3 months prevented loss of NO activity by saving nitrite in the PAOD study. This effect was less pronounced in the CAD study. On the other hand, Arg infusion in high dosage to children reduced reabsorption of nitrite, which is mediated by renal CA isoforms. In cardiovascular diseases such as PAOD and CAD, long-term Arg supplementation may save nitrite by improving CA-dependent nitrite reabsorption in the kidney. The optimum oral dosage of Arg remains to be established in these and other diseases associated with NO-related dysfunction. Further studies are needed investigating dose–response relationships between Arg and its different methylated analogs including ADMA, as well as its homolog homoarginine following oral and i.v. administration. This is because homoarginine recently emerged as a cardiovascular risk factor (März et al. 2010; Pilz et al. 2015) and it may antagonize the actions both of Arg (van der Zwan et al. 2013) and of ADMA (Tsikas and Kayacelebi 2014).

Acknowledgments The authors thank F.-M. Gutzki for performing GC–MS and GC–MS/MS analyses. We thank Prof. H. Hecker from the Institute of Biometry of the Hannover Medical School for the valuable help in performing the statistical analyses of the present study. We also thank the anonymous reviewers who helped improve our article.

Compliance with ethical standards

Conflict of interest The authors report no relationships that could be construed as a conflict of interest.

Ethical standard All studies reported here were approved by the local Ethics Committees. All adult participants and the parents of the children gave their written informed consent prior to enrollment.

References

Aamand R, Dalsgaard T, Jensen FB, Simonsen U, Roepstorff A, Fago A (2009) Generation of nitric oxide from nitrite by carbonic anhydrase: a possible link between metabolic activity and vasodilation. Am J Physiol Heart Circ Physiol 297:H2068–H2074

Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P (2003) Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively

metabolized by dimethylarginine dimethylaminohydrolase. Arterioscler Thromb Vasc Biol 23:1455–1459

Ahmetaj-Shala B, Kirkby NS, Knowles R, Al'Yamani M, Mazi S, Wang Z, Tucker AT, Mackenzie L, Armstrong PC, Nüsing RM, Tomlinson JA, Warner TD, Leiper J, Mitchell JA (2015) Evidence that links loss of cyclooxygenase-2 with increased asymmetric dimethyl arginine: novel explanation of cardiovascular side effects associated with anti-inflammatory drugs. Circulation 131:633–642

Bode-Böger SM (2006) Effect of arginine supplementation on NO production in man. Eur J Clin Pharmacol 62:91–99

Bode-Böger SM, Böger RH, Creutzig A, Tsikas D, Gutzki FM, Alexander K, Frölich JC (1994) L-arginine infusion decreases peripheral arterial resistance and inhibits platelet aggregation in healthy humans subjects. Clin Sci 87:303–310

Bode-Böger SM, Böger RH, Alfke H, Heinzel D, Tsikas D, Creutzig A, Alexander K, Frölich JC (1996) L-Arginine induces nitric oxide-dependent vasodilation in patients with critical limb ischemia. A randomized, controlled study. Circulation 93:85–90

Bode-Böger SM, Böger RH, Löffler M, Tsikas D, Brabant G, Frölich JC (1999) L-Arginine stimulates NO-dependent vasodilation in healthy humans—effects of somatostatin pretreatment. J Invest Med 47:43–50

Böger RH (2004) Asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase, explains the "L-arginine paradox" and acts as a novel cardiovascular risk factor. J Nutr 134:2842S–2847S

Böhmer A, Gambaryan S, Tsikas D (2014) Human blood samples lack nitric oxide synthase activity. Platelets. doi:10.3109/09537104.2014.974024

Brosnan ME, Brosnan JT (2004) Renal arginine metabolism. J Nutr 134:2791S–2895S

Chobanyan-Jürgens K, Schwarz A, Böhmer A, Beckmann B, Gutzki FM, Michaelsen JT, Stichtenoth DO, Tsikas D (2012) Renal carbonic anhydrases are involved in the reabsorption of endogenous nitrite. Nitric Oxide 26:126–131

Fitzgerald DJ, Fitzgerald GA (2013) Historical lessons in translational medicine: cyclooxygenase inhibition and P2Y12 antagonism. Circ Res 112:174–194

Förstermann U, Sessa WC (2012) Nitric oxide synthases: regulation and function. Eur Heart J 33:829–837

Gladwin MT, Raat NJ, Shiva S, Dezfulian C, Hogg N, Kim-Shapiro DB, Patel RP (2006) Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. Am J Physiol Heart Circ Physiol 291:H2026–H2035

Hu SD, Li XL, Rezaei R, Meininger CJ, McNeal CJ, Wu G (2015) Safety of long-term dietary supplementation with L-arginine in pigs. Amino Acids 47:925–936

Innocenti A, Vullo D, Scozzafava A, Supuran CT (2008) Carbonic anhydrase inhibitors: inhibition of mammalian isoforms I-XIV with a series of substituted phenols including paracetamol and salicylic acid. Bioorg Med Chem 16:7424–7428

Kanno K, Hirata Y, Emori R, Ohta K, Eguchi S, Imai T, Marumo F (1992) L-Arginine infusion induces hypotension and diuresis/natriuresis with concomitant increased urinary excretion of nitrite/nitrate and cyclic GMP in humans. Clin Exp Pharmacol Physiol 19:619–625

Kayacelebi AA, Willers J, Pham VV, Hahn A, Schneider JY, Rothmann S, Frölich JC, Tsikas D (2015a) Plasma homoarginine, arginine, asymmetric dimethylarginine and total homocysteine interrelationships in rheumatoid arthritis, coronary artery disease and peripheral artery occlusion disease. Amino Acids. doi:10.1007/s00726-015-1915-3

Kayacelebi AA, Langen J, Weigt-Usinger K et al (2015b) Biosynthesis of homoarginine (hArg) and asymmetric dimethylarginine (ADMA) from acutely and chronically administered free L-arginine in humans. Amino Acids. doi:10.1007/s00726-015-2012-3

Keimer R, Stutzer FK, Tsikas D, Troost R, Gutzki FM, Frölich JC (2003) Lack of oxidative stress during sustained therapy with isosorbide dinitrate and pentaerythrityl tetranitrate in healthy humans: a randomized, double-blind crossover study. J Cardiovasc Pharmacol 41:284–292

Kielstein A, Tsikas D, Galloway GP, Mendelson JE (2007) Asymmetric dimethylarginine (ADMA)—a modulator of nociception in opiate tolerance and addiction? Nitric Oxide 17:55–59

Lachmuth JY (2007) The APAV pilot study. L-Arginine in patients with peripheral arterial occlusive disease. PhD thesis. Hannover Medical School, Hannover. http://d-nb.info/991618793/34

Langen J, Kayacelebi AA, Beckmann B, Weight-Usinger K, Carmann C, Hörster I, Lilienthal E, Richter-Unruh, Tsikas D, Lücke T (2015) Homoarginine (hArg) and asymmetric dimethylarginine (ADMA) in short stature children without and with growth hormone deficiency: hArg and ADMA are involved differently in growth in the childhood. Amino Acids

Lundberg JO, Govoni M (2004) Inorganic nitrate is a possible source for systemic generation of nitric oxide. Free Radic Biol Med 37:395–400

Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG, Kim-Shapiro DB, Kozlov AV, Lancaster JR Jr, Lefer DJ, McColl K, McCurry K, Patel RP, Petersson J, Rassaf T, Reutov VP, Richter-Addo GB, Schechter A, Shiva S, Tsuchiya K, van Faassen EE, Webb AJ, Zuckerbraun BS, Zweier JL, Weitzberg E (2009) Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol 5:865–869

Lüneburg N, Xanthakis V, Schwedhelm E, Sullivan LM, Maas R, Anderssohn M, Riederer U, Glazer NL, Vasan RS, Böger RH (2011) Reference intervals for plasma L-arginine and the L-arginine:asymmetric dimethylarginine ratio in the Framingham Offspring Cohort. J Nutr 141:2186–2190

Mariotti F, Petzke KJ, Bonnet D, Szezepanski I, Bos C, Huneau JF et al (2013) Kinetics of the utilization of dietary arginine for nitric oxide and urea synthesis: insight into the arginine-nitric oxide metabolic system in humans. Am J Clin Nutr 97:972–979

März W, Meinitzer A, Drechsler C, Pilz S, Krane V, Kleber ME, Fischer J, Winkelmann BR, Böhm BO, Ritz E, Wanner C (2010) Homoarginine, cardiovascular risk, and mortality. Circulation 122:967–975

Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012

Ohrem HL, Schornick E, Kalivoda A, Ognibene R (2014) Why is mannitol becoming more and more popular as a pharmaceutical excipient in solid dosage forms? Pharm Dev Technol 19:257–262

Pilz S, Meinitzer A, Gaksch M, Grübler M, Verheyen N, Drechsler C, Hartaigh BÓ, Lang F, Alesutan I, Voelkl J, März W, Tomaschitz A (2015) Homoarginine in the renal and cardiovascular systems. Amino Acids. doi:10.1007/s00726-015-1993-2

Rassaf T, Feelisch M, Kelm M (2004) Circulating NO pool: assessment of nitrite and nitroso species in blood and tissues. Free Radic Biol Med 36:413–422

Rothmann S (2009) Pilot study on the effects of L-arginine in patients with coronary artery disease after angioplasty and method development for the determination of DMA in plasma as PFB derivative. PhD Thesis, Hannover Medical School, Hannover. http://d-nb.info/1007632712/34

Shi W, Meininger CJ, Haynes TE, Hatakeyama K, Wu G (2004) Regulation of tetrahydrobiopterin synthesis and bioavailability in endothelial cells. Cell Biochem Biophys 41:415–433

Sütö T, Losonczy G, Qiu C, Hill C, Samsell L, Ruby J, Charon N, Venuto R, Baylis C (1995) Acute changes in urinary excretion

of nitrite + nitrate do not necessarily predict renal vascular NO production. Kidney Int 48:1272–1277

Tajiri K, Myakawa H, Izumi N, Marumo F, Sato C (1995) Systemic hypotension and diuresis by L-arginine in cirrhotic patients with ascites: role of nitric oxide. Hepatology 22:1430–1435

Tousoulis D, Briasoulis A, Papageorgiou N, Tsioufis C, Tsiamis E, Toutouzas K, Stefanadis C (2011) Oxidative stress and endothelial function: therapeutic interventions. Recent Pat Cardiovasc Drug Discov 6:103–114

Tousoulis D, Papageorgiou N, Briasoulis A, Androulakis E, Charakida M, Tsiamis E, Stefanadis C (2012) Conflicting effects of nitric oxide and oxidative stress in chronic heart failure: potential therapeutic strategies. Heart Fail Rev 17:65–79

Tsikas D (1998) Application of gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry to assess in vivo synthesis of prostaglandins, thromboxane, leukotrienes, isoprostanes and related compounds in humans. J Chromatogr B 717:201–245

Tsikas D (2000) Simultaneous derivatization and quantification of the nitric oxide metabolites nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry. Anal Chem 72:4064–4072

Tsikas D (2008) A critical review and discussion of analytical methods in the L-arginine/nitric oxide area of basic and clinical research. Anal Biochem 379:139–163

Tsikas D (2015) Circulating and excretory nitrite and nitrate: their value as measures of nitric oxide synthesis, bioavailability and activity is inherently limited. Nitric Oxide 45:1–3

Tsikas D, Kayacelebi AA (2014) Do homoarginine and asymmetric dimethylarginine act antagonistically in the cardiovascular system? Circ J 78:2094–2095

Tsikas D, Böger RH, Sandmann J, Bode-Böger SM, Frölich JC (2000a) Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. FEBS Lett 478:1–3

Tsikas D, Gutzki FM, Böhme M, Fuchs I, Frölich JC (2000b) Solid- and liquid-phase extraction for the gas chromatographic-tandem mass spectrometric quantification of 2,3-dinor-thromboxane B2 and 2,3-dinor-6-oxo-prostaglandin F1 alpha in human urine. J Chromatogr A 885:351–359

Tsikas D, Schubert B, Gutzki FM, Sandmann J, Frölich JC (2003) Quantitative determination of circulating and urinary asymmetric dimethylarginine (ADMA) in humans by gas chromatography-tandem mass spectrometry as methyl ester tri (*N*-pentafluoropropionyl) derivative. J Chromatogr B 798:87–99

Tsikas D, Gutzki FM, Stichtenoth DO (2006) Circulating and excretory nitrite and nitrate as indicators of nitric oxide synthesis in humans: methods of analysis. Eur J Clin Pharmacol 62(Suppl 13):51–59

Tsikas D, Schwarz A, Stichtenoth DO (2010a) Simultaneous measurement of [^{15}N]nitrate and [^{15}N]nitrite enrichment and concentration in urine by gas chromatography mass spectrometry as pentafluorobenzyl derivatives. Anal Chem 82:2585–2587

Tsikas D, Wolf A, Mitschke A, Gutzki FM, Will W, Bader M (2010b) GC-MS determination of creatinine in human biological fluids as pentafluorobenzyl derivative in clinical studies and biomonitoring: inter-laboratory comparison in urine with Jaffé, HPLC and enzymatic assays. J Chromatogr B 878:2582–2592

Tsikas D, Niemann J, Flentje M, Schwarz A, Tossios P (2014) *N*-Acetylcysteine (NAC) inhibits renal nitrite and nitrate reabsorption in healthy subjects and in patients undergoing cardiac surgery: risk of nitric oxide (NO) bioavailability loss by NAC? Int J Cardiol 177:30–33

Tsikas D, Pham VV, Suchy MT, van de Ree MA, Huisman MV, Frölich JC, Princen HM, DALI-Study Group (2015) No effects of atorvastatin (10 or 80 mg/day) on nitric oxide, prostacyclin, thromboxane and oxidative stress in type 2 diabetes mellitus patients of the DALI study. Pharmacol Res 94:1–8

van der Zwan LP, Davids M, Scheffer PG, Dekker JM, Stehouver CDA, Teerlink T (2013) L-Homoarginine and arginine are antagonistically related to blood pressure in an elderly population: the Hoorn study. J Hypertens 31:1114–1123

West SG, Likos-Krick A, Brown P, Mariotti F (2005) Oral L-arginine improves hemodynamic responses to stress and reduces plasma homocysteine in hypercholesterolemic men. J Nutr 135:212–217

Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J et al (2009) Arginine metabolism and nutrition in growth, health and disease. Amino Acids 37:153–168

Yang Y, Wu ZL, Jia SC, Dahanayaka S, Feng S, Meininger CJ, McNeal CJ, Wu G (2015) Safety of long-term dietary supplementation with L-arginine in rats. Amino Acids. doi:10.1007/s00726-015-1992-3