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ABSTRACT

We recently found that renal carbonic anhydrase (CA) is involved in the reabsorption of inorganic nitrite (NO_2^-), an abundant reservoir of nitric oxide (NO) in tissues and cells. Impaired NO synthesis in the endothelium and decreased NO bioavailability in the circulation are considered major contributors to the development and progression of renal and cardiovascular diseases in different conditions including diabetes. Isolated human and bovine erythrocytic CAII and CAIV can convert nitrite to nitrous acid (HONO) and its anhydride N_2O_3 which, in the presence of thiols (RSH), are further converted to *S*-nitrosothiols (RSNO) and NO. Thus, CA may be responsible both for the homeostasis of nitrite and for its bioactivation to RSNO/NO. We hypothesized that enhanced excretion of nitrite in the urine may contribute to NO-related dysfunctions in the renal and cardiovascular systems, and proposed the urinary nitrate-to-nitrite molar ratio, i.e., $U_{NOx}R$, as a measure of renal CA-dependent excretion of nitrite. Based on results from clinical and experimental animal studies, here we report on a first evaluation of $U_{NOx}R$. We determined $U_{NOx}R$ values in preterm neonates, healthy children and adults, in children suffering from type 1 diabetes mellitus (T1DM) or Duchenne muscular dystrophy (DMD), in elderly subjects suffering from chronic rheumatic diseases, type 2 diabetes mellitus (T2DM), coronary artery disease (CAD) or peripheral arterial occlusive disease (PAOD). We also determined $U_{NOx}R$ values in healthy young men who ingested isosorbide dinitrate (ISDN), pentaerythrityl tetranitrate (PETN), or inorganic nitrate. In addition, we tested the utility of $U_{NOx}R$ in two animal models, i.e., the LEW.1AR1-*iddm* rat, an animal model of human T1DM, and the *APOE*3-Leiden.CETP* mice, a model of human dyslipidemia. Mean $U_{NOx}R$ values were lower in adult patients with rheumatic diseases (187) and in T2DM patients of the DALI study (74) as compared to healthy elderly adults (660) and healthy young men (1500). The intra- and inter-variability of $U_{NOx}R$ was of the order of 50% in young and elderly healthy subjects. $U_{NOx}R$ values were lower in black compared to white boys (314 vs. 483, P=0.007), which is in line with reported lower NO bioavailability in black ethnicity. Mean $U_{NOx}R$ values were lower in DMD (424) compared to healthy (730) children, but they were higher in T1DM children (1192). ISDN (3×30 mg) decreased stronger $U_{NOx}R$ compared to PETN (3×80 mg)

after 1 day (P=0.046) and after 5 days (P=0.0016) of oral administration of therapeutically equivalent doses. In healthy young men who ingested $NaNO_3$ (0.1 mmol/kg/d), $U_{NOx}R$ was higher than in those who ingested the same dose of NaCl (1709 vs. 369). In LEW.1AR1-*iddm* rats, mean $U_{NOx}R$ values were lower than in healthy rats (198 vs 308) and comparable to those in *APOE*3-Leiden.CETP* mice (151).

Keywords: Diabetes; Drugs; Health; Mass spectrometry; Nitric oxide reservoir; Renal carbonic anhydrase; Rheumatic disease

Abbreviations

ADMA	Asymmetric dimethylarginine
AQP	Aquaporin
BMI	Body mass index
CA	Carbonic anhydrase
CAD	Coronary artery disease
CysSNO	*S*-Nitrosocysteine
DALI	Diabetes Atorvastatin Lipid Intervention study
DMD	Duchenne muscular dystrophy
FSGS	Focal segmental glomerulosclerosis
GSNO	*S*-Nitrosoglutathione
ISDN	Isosorbide dinitrate
NAC	*N*-Acetylcysteine
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial NOS
iNOS	Inducible NOS
nNOS	Neuronal NOS
PAOD	Peripheral arterial occlusive disease
PAR	Peak area ratio

PETN	Pentaerythrityl tetranitrate
RSH	Thiols
RSNO	*S*-Nitrosothiols
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
U_{NOx}	Sum of urinary nitrate and nitrite
$U_{NOx}R$	Urinary nitrate-to-nitrite molar ratio
XOR	Xanthine oxidoreductase

Introduction

Nitric oxide (NO) possesses a wide spectrum of physiological functions that include regulation of blood pressure and platelet function (Moncada and Higgs 1993). 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 (Paolocci et al. 2001; Förstermann 2006; Landmesser et al. 2006; Heller et al. 2006). The role of the L-arginine/nitric oxide (L-Arg/NO) pathway (Fig. 1) and of oxidative stress in several diseases including diabetes mellitus, rheumatic and other autoimmune diseases has been frequently investigated. Yet, results are contradictory and underlying mechanisms are unresolved. Previously, we described a spontaneous animal model of human type 1 diabetes mellitus (T1DM), the LEW.1AR1-*iddm* rat (Lenzen et al. 2001), and demonstrated its suitability for delineating mechanisms involved in the development of autoimmune diabetes (Jörns et al. 2014). In the present study, we used this model to investigate the role of the L-Arg/NO pathway and of oxidative stress in the T1DM situation.

Nitrate is a major and nitrite a minor metabolite of NO (Tsikas 2008). In addition to the L-Arg/NO pathway, foods, water, air and pharmaceuticals may also contribute to nitrate and nitrite measured in biological samples (Fig. 1). Under certain standardized conditions,

most notably overnight fasting, avoidance of nitrate-rich diet and use of diuretics (Sütö et al. 1995; Rhodes et al. 1995; Baylis and Vallance 1998; Tsikas 2015), circulating and urinary nitrate are useful measures of systemic and whole-body NOS activity, respectively. In humans, circulating nitrite is a measure of endothelial NOS (eNOS) activity (Kleinbongard et al. 2003, 2006). Urinary 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ (15(*S*)-8-*iso*-$PGF_{2\alpha}$) is a biomarker of oxidative stress (Pham et al. 2009). In urine of patients with rheumatic diseases, nitrite correlated with 3-nitrotyrosine suggesting nitrite as a biomarker of nitrosative stress (Pham et al. 2009). Based on the sum of urinary nitrate and nitrite (U_{NOx}) as measured by the Griess assay after reduction of nitrate to nitrite, previous studies in conscious rats (Sütö et al. 1995) and anaesthetized dogs (Godfrey and Majid 1998) indicated that urinary nitrate/nitrite excretion depends predominantly on tubular handling. Especially, intravenous administration of the carbonic anhydrase (CA) inhibitor acetazolamide to conscious rats increased U_{NOx} by a factor of almost 3, strongly suggesting involvement of renal CA in the handling of nitrate/nitrite excretion/reabsorption (Sütö et al. 1995). In humans, we have recently shown that renal CA isoforms are involved in the reabsorption of inorganic nitrite (NO_2^-) and to a lesser extent of inorganic nitrate (ONO_2^-) (Tsikas and Chobanyan-Jürgens 2010; Chobanyan-Jürgens et al. 2012a; Tsikas et al. 2014; Schneider et al. 2015). This newly discovered role of renal CA isoforms is of particular importance, because nitrite is an important and abundant reservoir of NO in tissues and cells including erythrocytes (Gladwin et al. 2006). Nitrite, but not nitrate, can be reduced to NO by several proteins and enzymes including hemoglobin and xanthine oxidoreductase (XOR) (Fig. 1). Furthermore, in vitro isolated bovine erythrocytic CAII treated with the CA inhibitors dorzolamide and acetazolamide has been reported to enhance NO formation from nitrite (Aamand et al. 2009). However, we (Hanff et al. 2015; Zinke et al. 2015) and others (Andring et al. 2018) found that CAII and CAIV do not reduce nitrite to NO in the presence or absence of dorzolamide or acetazolamide. We demonstrated that human and bovine CA isoforms can convert nitrite into *S*-nitrosoglutathione (GSNO) and *S*-nitrosocysteine (CysSNO) (Hanff et al. 2015; Zinke et al. 2015). GSNO and CysSNO possess NO-related biological activities including vasodilation and inhibition of platelet aggregation (Fig. 1). CysSNO is a potent NO

donor and a strong inhibitor of platelet aggregation acting via cGMP-dependent and cGMP-independent mechanisms (Tsikas et al. 1999). CysSNO and GSNO are potent endogenous *S*-nitrosylating species.

The CA inhibitor acetazolamide was found to increase the excretion of nitrite in healthy humans at oral therapeutic doses (5 mg/kg), suggesting a role of renal CA in the reabsorption of nitrite (Chobanyan-Jürgens et al. 2012a) (Fig. 1). We also found that *N*-acetylcysteine (NAC) increased nitrite and nitrate excretion in the urine, suggesting that NAC is an inhibitor of renal CA isoforms (Tsikas et al. 2014). In vitro, paracetamol (acetaminophen) has been shown to inhibit the CO_2 hydration activity of several human CA isozymes (K_i range, 4 μM to 800 μM) (Innocenti et al. 2008).

In consideration of the potential role of renal CA isoforms in maintaining NO homeostasis, we hypothesized that enhanced excretion of nitrite in the urine may be an additional, not yet considered factor affecting NO homeostasis in the circulation. We recently proposed the urinary nitrate-to-nitrite molar ratio $U_{NOx}R$ as a measure of renal CA-dependent reabsorption of urinary nitrite in humans (Schneider et al. 2015) (Fig. 1). The whole-body NO synthesis can be expressed as the sum of the concentration of nitrate and nitrite in urine: [NO] = $[nitrate]_U$ + $[nitrite]_u$. Because $[nitrate]_U$ is much higher than $[nitrite]_u$ in non-infected urine, and urinary nitrate's reabsorption is only marginally dependent upon renal CA isoforms (see below), whole-body NO synthesis can be expressed in approximation solely by the urinary excretion of nitrate: [NO] ≈ $[nitrate]_U$. In contrast, urinary nitrite is reabsorbed in the kidney mainly by a mechanism involving renal CA isoforms (Tsikas and Chobanyan-Jürgens 2010; Chobanyan-Jürgens et al. 2012a; Tsikas et al. 2014; Schneider et al. 2015). In theory, the concentration of nitrite in the urine could be used as a measure of a nitrite-dependent renal CA activity. However, as nitrate and nitrite cannot be considered independent of each other, we divided the $[nitrate]_U$ by the $[nitrite]_U$ and proposed the formula $U_{NOx}R$ = $[nitrate]_U/[nitrite]_u$ to estimate CA-dependent reabsorption of nitrite in the kidney. High $U_{NOx}R$ values would express high nitrite reabsorption and thus high CA activity. Low $U_{NOx}R$ values would express low nitrite reabsorption and low CA activity.

In the present study, we determined the $U_{NOx}R$ in LEW.1AR1-*iddm* rats (i.e., model of T1DM), in *APOE*3-Leiden.CETP* mice (i.e., model of dyslipidemia), in adults with type 2 diabetes mellitus (T2DM), in adults suffering from rheumatic diseases, peripheral artery occlusive disease (PAOD) or coronary artery disease (CAD), in healthy adults and in healthy young black and white boys. The T1DM and T2DM animal models are discussed in more detail in the next section. We also determined the $U_{NOx}R$ in healthy young subjects before and after oral intake of therapeutically equivalent doses of the organic nitrates isosorbide dinitrate (ISDN) or pentaerythrityl tetranitrate (PETN), which are known to be metabolized to inorganic nitrite and nitrate which are found both in blood and in urine (Keimer et al. 2003) (Fig. 1). The baseline data reported and discussed in the present paper are summarized in Table 1. The potential utility and limitations of $U_{NOx}R$ in experimental and clinical studies are discussed. Eventually, we address the presumably CA-dependent bioactivation of inorganic nitrite to potential mutagenic and cancerogenic nitrosamines such as *N*-nitrososarcosine (Fig. 1).

Methods

Human studies

$U_{NOx}R$ in healthy subjects who took acetazolamide

Acetazolamide is a strong CA inhibitor (IC_{50} 0.1 µM with respect to esterase activity). A 44-years old healthy male volunteer took orally acetazolamide (a 500-mg Diamox retard capsule, Goldshield Pharmaceuticals Ltd, U.K.) according to previously reported studies (Tsikas and Chobanyan-Jürgens 2010; Chobanyan-Jürgens et al. 2012a). The volunteer did not take any drugs in the preceding two weeks and was not fasting overnight, but restrained from nitrite/nitrate-rich foods and beverages for 12 h before and during the study. In the morning (8 a.m.), the volunteer was given orally one capsule acetazolamide

corresponding to a dose of about 5 mg/kg bodyweight. First, the volunteer emptied his bladder and collected the first urine specimen. Immediately after collection of the 0-h urine sample, acetazolamide was taken with 200 mL of drinking water. Urine samples were collected in polypropylene tubes which were immediately closed, put on ice and the urine pH was measured. Then urine samples were aliquoted in 1-mL proportions and carbonate was measured at the same day. Nitrite, nitrate and creatinine were measured on next day after thawing the samples which had been stored at -20 °C. Fresh and thawed urine samples were centrifuged (800×g, 4 °C, 5 min) before analytical measurements.

$U_{NOx}R$ in healthy young men and effects of organic nitrates

In a previous randomized, double-blind, crossover study (Keimer et al. 2003), 18 healthy non-obese young men (mean age, 26 years) collected urine samples for 24 hours at baseline and 1 and 5 days after daily oral administration of therapeutically relevant doses of isosorbide dinitrate (ISDN, 3×30 mg per day) or pentaerythrityl tetranitrate (PETN, 3×80 mg per day) with an in-between interval of three weeks. By using the previously measured urinary nitrate and nitrite concentrations (Keimer et al. 2003) we calculated the $U_{NOx}R$ values before (day 0) and after drug ingestion (day 1 and day 5) (see Table 1).

Effects of beta-blockers on $U_{NOx}R$ in healthy young men

In a previous double-blind, randomized, cross-over study (Fahlbusch et al. 2004), 17 healthy men received antihypertensive doses of the beta-blockers carvedilol (25 mg b.i.d.), metoprolol (100 mg b.i.d.) or placebo for 6 days. The previously measured urinary nitrate and nitrite concentrations (Fahlbusch et al. 2004) were used to calculate the $U_{NOx}R$ values before (day 0) and after drug ingestion (see Table 1).

$U_{NOx}R$ in hypogonadal men and effects of testosterone treatment

Previously (Leifke et al. 2008, Tsikas and Kinzel 2017), we investigated the effects of normalization of plasma testosterone levels in ten hypogonadal men on plasma levels and urinary excretion of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor

(Tsikas 2008). In urine samples of eight men of that study the concentration of nitrate and nitrite was determined and the $U_{NOx}R$ values were calculated (see Table 1).

$U_{NOx}R$ in healthy elderly subjects and patients suffering from rheumatic diseases

By using the measured urinary nitrate and nitrite concentrations of a previously reported study (Pham et al. 2009) we calculated the U_{NOxR} values in healthy subjects (*n*=41; 16 women, 25 men; mean age, 47 years) and in patients with chronic rheumatic diseases (*n*=27; 18 women, 10 men; mean age, 55 years) (see Table 1).

$U_{NOx}R$ in humans suffering from T2DM

The Diabetes Atorvastatin Lipid Intervention (DALI) study is a randomised double-blind, placebo-controlled, multicentre parallel-group study on 217 patients with T2DM and diabetic dyslipidemia conducted in the Netherlands (The DALI Study Group 2001). In T2DM patients who did not receive atorvastatin we measured the urinary excretion of nitrate and nitrite and calculated the $U_{NOx}R$ values. Patients' age and diabetes duration were (mean±SD) 59.6±7.7 years and 10.1±7.5 years, respectively (Tsikas et al. 2015) (see Table 1).

$U_{NOx}R$ in preterm neonates

Previously (Buck et al. 2017), we measured urinary nitrate and nitrite concentrations in preterm neonates (34 girls, 42 boys) with a gestational age of 23+6 to 36+1 weeks and calculated the $U_{NOx}R$ values (Table 1).

$U_{NOx}R$ in healthy black and white boys from South Africa

Cardiovascular disease occurs earlier in black ethnicity than in white counterparts (Schutte et al. 2017). We designed a study to include children at the earliest possible age whereby the child themselves had the choice of study participation. Female sex was excluded due to potential early onset menses and hormonal influences. The age range of 6-8 years was selected and children were recruited from the foundation phase in pre-primary and primary

schools. The selection was made in schools with demographically equal black and white ethnicity and to account for socioeconomic comparison. In this study, we found that black boys have a compromised vasculature in terms of increased arterial stiffness, diastolic blood pressure, and carotid wall thickness and advanced glycation end products (Mokwatsi et al. 2017). We hypothesized that the measurement of $U_{NOx}R$ values in these black and white boys would contribute to a better understanding of the early onset of cardiovascular ageing in black ethnicity. In urine samples collected previously we newly determined nitrate, nitrite and creatinine concentrations as described previously (Hanff et al. 2017) and calculated the $U_{NOx}R$ values (Table 1).

Animal studies

$U_{NOx}R$ in the *LEW.1AR1-iddm* rat as model of human T1DM

Diabetes manifested in the animals (n=5) at day 66±4 of life with an average blood glucose concentration of 14.8±1.3 mM. Thereafter, a chronic diabetic metabolic state was maintained for around 4 months (109±14 days) during which blood glucose values were maintained within a range of 13-23 mM through insulin supplementation by implantation of a slow-release insulin pellet (Linplant®, Lin Shin Canada Ltd, Toronto, Canada). In urine samples collected from these chronically diabetic rats (blood glucose, 18.1±2.7 mM; n=5) and non-diabetic control rats (blood glucose, 5.1±2.7 mM; n=5) of either sex, we determined representative biomarkers of the L-Arg/NO pathway, i.e., nitrite, nitrate, $U_{NOx}R$, and ADMA, as well as the oxidative stress biomarker 15(S)-8-*iso*-$PGF_{2\alpha}$. Creatinine excretion during the same period of time did not differ between the groups (1.42±0.22 vs 1.36±0.41 µmol, P=0.916). All data reported above are given as mean±SD. Biomarker urinary excretion rates in this and in the other studies described in this work were corrected for creatinine excretion and are expressed as nmol or µmol analyte per mmol creatinine.

$U_{NOx}R$ in *APOE*3-Leiden.CETP* mice

The *APOE*3-Leiden.CETP* mice are a well-established mouse model for familial dysbetalipoproteinemia with human-like lipoprotein metabolism and atherosclerosis development which respond in a human-like manner to both lipid-lowering and HDL-raising drugs like statins, fibrates and niacin used in the treatment of cardiovascular diseases (Zadelaar et al. 2007; van der Hoogt et al. 2007; van der Hoorn et al. 2008; de Haan et al. 2008; Westerterp et al. 2006). We determined urinary nitrate and nitrite concentrations and calculated the U_{NOxR} values in urine samples of a previously reported study (Berbée et al. 2013) in dyslipidemic female *APOE*3-Leiden.CETP* mice treated with resveratrol, atorvastatin or with a combination of resveratrol and atorvastatin.

In a second *APOE*3-Leiden.CETP* mice study, we investigated the effect of oral gavage of ritonavir (ritonavir, 60 mg/kg/d) alone and in combination with lopinavir (lopinavir/ritonavir, 40/10 mg/kg/d), of atazanavir (60 mg/kg/d) or placebo (vehicle PEG-400) for 16 days. Pooled urine samples from 2-3 mice in metabolic cages were collected during the night at three different time points during the study.

The experiments in *APOE*3-Leiden.CETP* mice were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Research (TNO).

Analytical methods

The concentrations of nitrite, nitrate, ADMA, 15(*S*)-8-*iso*-$PGF_{2\alpha}$, creatinine and bicarbonate in urine samples of the studies described in this work were determined by previously reported fully validated GC-MS and GC-MS/MS methods (Tsikas 2000; Tsikas et al. 2003a, b, 2010; Hanff et al. 2017). In the human studies and where possible in the animal studies reported in the present work, precautions were taken to prevent both loss of nitrite and artefactual formation of nitrite from nitrate by bacterial nitrate reductases. These precautions included avoidance of urine acidification, storage of urine during collection in a refrigerator at 4 – 8 °C, and storage at -20 °C until analysis. For collection of human urine over 24 h polypropylene bottles containing 1 mM each of 4-hydroxy-TEMPO and EDTA were used as radical and metal ions scavengers, respectively.

Statistical analyses

Data are presented as mean±SD, mean±SEM or median [25th-75th interquartile] as indicated in the text. Statistical analysis was performed using the Mann-Whitney test (and unpaired *t*-test for unpaired analysis) and the Wilcoxon test (and paired *t*-test for paired analysis). If not otherwise stated Spearman correlation was performed. Statistical significance was assumed for $P<0.05$. Data analysis was performed using GraphPad Prism 5.

Results

The results of the studies reported and discussed in the present work are summarized in Table 1 and presented in the Figures that follow.

Human studies

$U_{NOx}R$ in healthy subjects taken acetazolamide

In the urine samples, the concentration of nitrate, nitrite and carbonate changed concomitantly upon ingestion of the CA inhibitor acetazolamide (Fig. 2A). In the time window of 0 – 3 h with maximum inhibition of CA activity, there were close correlations between carbonate and nitrite (Spearman $r=0.893$, $P=0.012$), and between nitrate and nitrite ($r=0.857$, $P=0.024$), while the correlation between carbonate and nitrate failed barely statistical significance ($r=0.75$, $P=0.066$). The urinary excretion of nitrate and nitrite and the $U_{NOx}R$ value correlated with the pH value of the urine samples (Fig. 2B-D). Urinary nitrate ($r=0.991$) and nitrite ($r=0.998$) increased moderately and linearly with increasing urinary pH until the value of 7.31. In the very narrow pH range of 7.38 to 7.45, urinary nitrate decreased suddenly and linearly ($r=-0.974$), while urinary nitrite increased abruptly and linearly ($r=0.922$). In this pH range, the $U_{NOx}R$ value decreased also suddenly (Fig. 2D). Considering the entire time window (24 h), the $U_{NOx}R$ value correlated inversely with the

creatinine-corrected excretion of carbonate (r=-0.955, P<0.0001) and nitrite (r=-1.000, P<0.0001). $U_{NOx}R$ correlated inversely with the urinary pH (r=-0.997, P<0.0001), whereas creatinine-corrected nitrate excretion did not correlate with $U_{NOx}R$ (r=-0.358, P=0.313). These observations suggest that $U_{NOx}R$ is a useful parameter to measure renal CA-dependent nitrite reabsorption in humans (see Fig. 1).

Effects of organic nitrates on $U_{NOx}R$ in healthy young men

In 18 healthy non-medicated young men of a previously reported study (Keimer et al. 2003), the baseline $U_{NOx}R$ values varied by about 60%, but the average baseline $U_{NOx}R$ value did not differ significantly on day 1 and day 21 (P=0.371; Fig. 3A). The baseline creatinine-corrected nitrate and nitrite excretion rates were (mean±SEM) 97.6±13.2 µmol/mmol and 74.3±9.3 nmol/mmol on day 1, and 109±22 µmol/mmol and 127±38 nmol/mmol on day 21, respectively (Table 1). At baseline, creatinine-corrected excretion of nitrite and nitrate did not correlate with each other in the PETN and ISDN groups (r=0.362, P=0.139 and r=0.257, P=0.303, respectively). Creatinine-corrected excretion of nitrite (r=-0.694, P=0.001 and r=-0.598, P=0.009) but not of nitrate (r=0.352, P=0.152 and r=0.416, P=0.086) did correlate with $U_{NOx}R$ in the PETN and ISDN groups, respectively. Intake of PETN or ISDN resulted in decreases of the $U_{NOx}R$ values (Fig. 3B). Thus, the $U_{NOx}R$ values were (mean±SEM) 1570±233 at baseline and decreased to 801±132 after 1 day and to 599±44 after 5 days of PETN administration. The $U_{NOx}R$ values were (mean±SEM) 1403±188 at baseline and decreased to 483±66 after 1 day and to 383±30 after 5 days of ISDN administration (Table 1). For PETN and ISDN the $U_{NOx}R$ values between baseline and day 1 (P=0.004 and P=0.0002, respectively) or day 5 (P=0.0005 and P<0.0001, respectively) were statistically significantly different (two-tailed paired t test). For both drugs the differences were not significant when comparing day 1 with day 5 of drug administration. The $U_{NOx}R$ values did not differ between the two groups (P=0.681) at baseline, but $U_{NOx}R$ values did significantly differ after 1 day (P=0.046) and after 5 days (P=0.002) of treatment. These observations indicate that both, PETN and ISDN, affected the renal CA-dependent nitrite excretion in the healthy young men at the doses used.

Effects of beta-blockers on $U_{NOx}R$ in healthy young men

In 15 healthy non-medicated young men of a previously reported study (Fahlbusch et al. 2004), the creatinine-corrected nitrate excretion rates were (mean±SEM) 90.5±15.6 µmol/mmol in the placebo group (Table 1), 57.6±9.7 µmol/mmol in the metoprolol group and 62.2±15.4 µmol/mmol in the carvedilol group. The corresponding creatinine-corrected nitrite excretion rates were 291±55.4 nmol/mmol (Table 1), 193.9±25.4 nmol/mmol and 243.9±43.8 nmol/mmol. The calculated $U_{NOx}R$ values in the placebo group (406±61) (Table 1) did not differ from those in the metoprolol group (358±67, P=0.54) and the carvedilol group (314±52, P=0.42). These observations suggest that neither metoprolol nor carvedilol affected the renal CA-dependent reabsorption of nitrite in the healthy young men at the therapeutical doses used.

Relationship between $U_{NOx}R$ and blood pressure in PAOD

Nitrite is reabsorbed in the kidneys in part due to renal CA activity. We proposed the urinary nitrate-to-nitrite molar ratio $U_{NOx}R$ as a measure of this type of CA activity and NO bioavailability in the circulation (Fig. 1). Loss of nitrite, an abundant reservoir of NO in the circulation (Gladwin et al. 2006), may contribute to the development and/or progression of NO-related cardiovascular dysfunction in various diseases. Strong supporting evidence of this provides our observation that at baseline both systolic and diastolic pressure values correlate inversely with the $U_{NOx}R$ values measured in 40 PAOD patients divided into two groups consisting each of 20 patients (Schneider et al. 2015) (Fig. 4). Considering the baseline values of the whole collective, creatinine-corrected excretion of nitrate and nitrite correlated with each other (r=0.481, P=0.002). $U_{NOx}R$ correlated inversely with the creatinine-corrected excretion of nitrite (r=-0.713, P<0.0001), but did not correlate with creatinine-corrected excretion of nitrate (r=0.150, P=0.357). In the ARG group (patients received orally L-arginine for three months), creatinine-corrected excretion of nitrite correlated inversely with $U_{NOx}R$ at baseline (r=-0.862, P<0.0001) and after treatment (r=-0.578, P=0.0076). In the ARG group, creatinine-corrected excretion of nitrate did not

correlate with $U_{NOx}R$ at baseline (r=0.045, P<0.85) and after treatment (r=0.311, P=0.1814). In the PLA group (patients received orally mannitol for three months), creatinine-corrected excretion of nitrite correlated inversely with $U_{NOx}R$ at baseline (r=-0.643, P=0.0022) and after treatment (r=-0.891, P<0.0001). In the PLA group, creatinine-corrected excretion of nitrate did not correlate with $U_{NOx}R$ at baseline (r=0.404, P=0.0774), but did correlate positively after treatment (r=0.681, P=0.001). L-Arginine supplementation to the PAOD patients (ARG group) for three months did not change $U_{NOx}R$ (461 [311-678] vs. 545 [311-594] P=0.858). Also, placebo (mannitol) supplementation to the PAOD patients (PLA group) for three months did not result in considerable decrease in $U_{NOx}R$ (342 [266-498] vs. 316 [157-441] P=0.409). $U_{NOx}R$ did not differ in the groups at baseline (P=0.1632), but did differ after treatment with L-arginine or placebo (P=0.024).

In a similar study, 60 patients suffering for CAD were treated with L-arginine (ARG group, n=31) or mannitol as placebo (PLA group, n=29) for three and six months (Schneider et al. 2015) (Table 1). Considering all data points available (n=168), creatinine-corrected excretion of nitrite correlated positively with the creatinine-corrected excretion of nitrate (r=0.457, P<0.0001) and inversely with $U_{NOx}R$ (r=-0.777, P<0.001). Creatinine-corrected excretion of nitrate failed to correlate with $U_{NOx}R$ (r=0.143, P=0.064). In the PLA group, $U_{NOx}R$ decreased considerably after three and six months (409 [237-777] vs. 273 [121-438] P=0.0036 and 273 [154-361] P=0.0028, respectively). In the ARG group, $U_{NOx}R$ did not change after three and six months (406 [225-708] vs. 430 [250-570] P=0.495 and vs. 300 [217-447] P=0.284, respectively). Obviously, L-arginine supplementation avoided a decrease of $U_{NOx}R$ over three and six months.

In the PAOD and CAD studies, patients received orally either L-arginine or mannitol at high dosages (Schneider et al. 2015). L-Arginine, D-arginine and mannitol are known to act on the proximal tubule and to affect bicarbonate reabsorption (DuBose and Lucci 1983; Battle and Chan 1989; Sütö et al. 1995). The PAOD and CAD patients of our studies suffered from additional diseases including diabetes mellitus, and they received chronically many drugs including organic nitrates. Although oral bioavailability of mannitol and its subsequent excretion in urine are relatively low (Sequeira et al. 2012), the situation in the

PAOD and CAD studies is very complex and nitrite-reabsorption may have been affected by many different factors in the patients (see also below).

$U_{NOx}R$ in hypogonadal men and effects of testosterone treatment

In the urine samples of eight hypogonadal men of a previously reported study (Leifke et al. 2008), the baseline creatinine-corrected nitrate and nitrite excretion rates were determined to be 66.5 [53.6-84.5] µmol/mmol and 0.56 [0.41-0.75] µmol/mmol, respectively; the corresponding $U_{NOx}R$ value was calculated to be 123±33 (Tsikas and Kinzel 2017; Table 1). These baseline $U_{NOx}R$ levels are low, indicating impaired renal CA-dependent reabsorption of nitrite in the hypogonadal men. At baseline, $U_{NOx}R$ correlated positively with plasma testosterone (r=0.72, P=0.044) (Tsikas and Kinzel 2017). After pharmaceutical normalization of plasma testosterone levels with transdermal testosterone, the creatinine-corrected nitrate and nitrite excretion rates were lower, i.e., 49.8 [41.1-58.6] µmol/mmol and 0.31 [0.19-0.55] nmol/mmol, respectively. The corresponding $U_{NOx}R$ value was calculated to be 156±60. Yet, the decreases in nitrate (P=0.065) and nitrite excretion (P=0.141) and the increase in $U_{NOx}R$ were not statistically different (P=0.505). Before and after testosterone treatment, creatinine-corrected excretion of nitrate and nitrite correlated with each other (r=0.832, P=0.01 and r=0.858, P=0.006, respectively). At baseline, creatinine-corrected excretion of nitrate and nitrite correlated inversely with $U_{NOx}R$: r=-0.680, P=0.063 and r=-0.891, P=0.003, respectively. After treatment, creatinine-corrected excretion of nitrate and nitrite correlated inversely with $U_{NOx}R$: r=-0.660, P=0.075 and r=-0.867, P=0.005, respectively. These correlations did not change when considering all data together: r=0.855, P<0.0001 for nitrate vs. nitrite; r=-0.654, P=0.006 for nitrate vs. $U_{NOx}R$; and r=-0.836, P<0.0001 for nitrite vs. $U_{NOx}R$. Interestingly, the $U_{NOx}R$ values correlated inversely with the creatinine-corrected urinary excretion of ADMA (Tsikas and Kinzel 2016), both at baseline (r=-0.71, P=0.049) and after testosterone treatment (r=-0.75, P=0.032).

$U_{NOx}R$ in healthy non-medicated elderly subjects and in patients suffering from rheumatic diseases

As reported earlier (Pham et al. 2009), patients with rheumatic diseases were found to have higher creatinine-corrected excretion rates of nitrite (1100±300 vs. 190±20 nmol/mmol) but not of nitrate (105±13 vs 106±12 µmol/mmol, *P*=0.80) when compared to healthy subjects of comparable age (Table 1). To evaluate potential biological variation and differences in gender and age, we determined the $U_{NOx}R$ values in a group of middle-aged healthy non-medicated men and women of a previously reported study (Pham et al. 2009). In 41 healthy subjects (16 females, 25 males; mean age, 47 years; range, 26 – 82 years), the $U_{NOx}R$ values did not differ (*P*=0.455) between men and women (mean±SD): 605±348 vs. 663±367 (Fig. 5A), but varied considerably within the groups by 58% and 55%, respectively. The $U_{NOx}R$ values of the whole group ranged between 166 and 1577 (mean±SD, 626±351) and varied on average by 56%. In this population, there was no correlation between the $U_{NOx}R$ values and the pH values of the urine samples (Fig. 5B) or age of the subjects (Fig. 5C). Patients with chronic rheumatic diseases had statistically significantly lower $U_{NOx}R$ values as compared to the middle-aged healthy subjects (mean±SEM; 187±22 vs. 660±72, *P*<0.0001), suggesting impaired renal CA-dependent excretion of nitrite in chronic rheumatic diseases. Among the rheumatic patients, the lowest $U_{NOx}R$ values were observed in those suffering from rheumatoid arthritis (138±34, *n*=10) and the highest in patients with undifferentiated arthritis (230±34, *n*=10); the difference between these subgroups did not reach statistical significance (Mann-Whitney test, *P*=0.089). Creatinine-corrected excretion of nitrate and nitrite correlated with each other in the rheumatic patients (*r*=0.379, *P*=0.047) and in the healthy subjects (*r*=0.335, *P*=0.032). $U_{NOx}R$ correlated more closely with the creatinine-corrected excretion of nitrate in the healthy subjects (*r*=0.608, *P*<0.0001 vs. *r*=0.340, *P*<0.0001 in patients), as well as with the creatinine-corrected excretion of nitrite in the patients (*r*=-0.612, *P*=0.001 vs. *r*=-0.479, *P*=0.002 in healthy subjects).

$U_{NOx}R$ in human type 2 diabetes mellitus

T2DM patients excrete considerably higher nitrate (on average 129 to 162 μmol/mmol creatinine) and nitrite (on average 2.3 to 3.9 nmol/mmol creatinine) amounts in the urine than healthy subjects (Tsikas et al. 2015). We calculated the $U_{NOx}R$ values using the urinary nitrate and nitrite concentrations measured in the previously reported study. We also investigated potential correlations between $U_{NOx}R$ and age, disease duration or inflammation markers, as well as the effects of the lipid-lowering drug atorvastatin in the T2DM patients in the DALI study (Tsikas et al. 2015).

$U_{NOx}R$ values were (mean±SEM) only 68.3±3.8 at baseline in the T2DM patients of the DALI study, indicating severely impaired CA-dependent nitrite reabsorption in the T2DM patients (Table 1). There was no correlation between $U_{NOx}R$ and age or diabetes duration (Fig. S1A, B; see Supplement). Creatinine-corrected excretion of nitrate and nitrite correlated with each other in the T2DM patients (r=0.322, P<0.0001). $U_{NOx}R$ correlated more closely with the creatinine-corrected excretion of nitrite (r=-0.610, P<0.0001) than of nitrate (r=-0.495, P<0.0001). At baseline, urinary nitrite and nitrate excretion and $U_{NOx}R$ did not correlate with the inflammation markers TNF_{α}, IL-1β, CRP (data not shown). Treatment of the T2DM patients with atorvastatin (10 mg/day or 80 mg/d) for 30 weeks did not result in changes of $U_{NOx}R$ (Fig. S1C; see Supplement). At the end of the treatment, CRP correlated weakly with urinary nitrate (r=0.150, P=0.039) and nitrite (r=-0.243, P=0.003) excretion, and with $U_{NOx}R$ (r=0.318, P=0.0001).

Postprandial $U_{NOx}R$ changes after ingestion of high-fat protein meals in overweight men

At three different occasions, in a cross-over design, we administered three high-fat meals to healthy overweighed men (n=10; BMI > 25 kg/m^2; waist circumference >94 cm). The meals were made of 1200 kcal from fat (70%) and sucrose (15%) and differed according to the nature of the dietary protein, which was either casein (CAS) or whey proteins, enriched (LAC) or not (WHE) with α-lactalbumin protein. Morning urine was discarded on the arrival at the clinical center; urine was sampled immediately before meal and then collected every

2 h for 6 h after the ingestion of the meal. The clinical study has been described in full elsewhere (Mariotti et al. 2015).

When considered all three high-fat meals together, $U_{NOx}R$ did not change upon ingestion; $U_{NOx}R$ values also did not change upon ingestion of each meal by the ten overweight men (Fig. S2; see Supplement) (Table 1). The baseline $U_{NOx}R$ values varied by about 50% on the three meal tests which were performed in a period of at least 6 weeks. Considering all data (n=124), urinary creatinine concentration correlated with $U_{NOx}R$ (r=0.594, P<0.0001). Creatinine-corrected excretion of nitrite and nitrate correlated with each other (r=0.614, P<0.0001) and with $U_{NOx}R$, yet in an opposite manner: r=-0.591, P<0.0001 for nitrite, and r=0.614, P=0.023 for nitrate.

$U_{NOx}R$ in preterm neonates

In preterm neonates (34 girls, 42 boys) with a gestational age of 23+6 to 36+1 weeks, we measured gender-independent $U_{NOx}R$ values of 210±14. The nitrate (283±85 µmol/mmol creatinine) and nitrite (4.3±10.7 µmol/mmol creatinine) excretion rates are among the highest we measured thus far in healthy adults (Table 1). Creatinine-corrected excretion of nitrite and nitrate correlated with each other (r=0.304, P=0.008). Creatinine-corrected excretion of nitrite (r=-0.873, P<0.0001), but not of nitrate (r=0.094, P=0.417), did correlate with $U_{NOx}R$.

Effect of L-arginine infusion on $U_{NOx}R$ in growth hormone deficiency

An indication for a possible effect of L-arginine, which acts on the proximal tubule of the nephron, on the reabsorption of urinary nitrite, has provided by the so called L-arginine test. We applied this clinical test to seven children suspected to have growth hormone deficiency. Infusion of L-arginine (0.5 g L-arginine/kg for 30 min) resulted in peak mM-concentrations of L-arginine in the plasma and in increases in the creatinine-corrected excretion of nitrate (1.1-fold) and nitrite (2-fold); $U_{NOx}R$ decreased by about 40% during the test (Schneider et al. 2015) (Table 1). Before starting the L-arginine infusion, creatinine-corrected nitrate and nitrite correlated with other (r=0.893, P=0.012). $U_{NOx}R$ did correlate

inversely with the creatinine-corrected excretion of nitrite (r=-0.893, P=0.012), but not of nitrate (r=-0.643, P=0.132). At the end of the L-arginine test, creatinine-corrected excretion of nitrate and nitrite correlated with each other (r=0.821, P=0.034), but neither nitrate nor nitrite excretion did correlate with $U_{NOx}R$.

$U_{NOx}R$ in healthy black and white boys

In urine samples of the age-matched black and white boys from South Africa collected in a previously reported study (Mokwatsi et al. 2017), we measured similar nitrite and creatinine concentrations but different nitrate concentrations (P=0.0007). These differences resulted in statistically significantly (P=0.007) higher $U_{NOx}R$ values in the white boys compared to the black boys (mean±SD): 483±314 vs. 314±155 (Table 1). Creatinine-corrected excretion rates of nitrate and nitrite correlated with each other in the white boys (r=0.383, P=0.014) and in the black boys (r=0.501, P=0.001). In the white boys, $U_{NOx}R$ correlated more closely with the creatinine-corrected excretion of nitrate (r=0.645, P<0.0001) than of nitrite (r=-0.348, P=0.026). In the black boys, $U_{NOx}R$ did not correlate with the creatinine-corrected excretion of nitrate (r=0.112, P=0.497), but did correlate closely with the creatinine-corrected excretion of nitrite (r=-0.723, P<0.0001). Urinary creatinine concentration correlated with $U_{NOx}R$ in the white boys (r=0.570, P=0.0001) and in the black boys (r=0.847, P<0.0001). Considering all data (n=80), urinary creatinine concentration correlated with $U_{NOx}R$ (r=0.714, P<0.0001).

Animal studies

$U_{NOx}R$ in the *LEW.1AR1-iddm* rat as model of human type 1 diabetes mellitus

To investigate whether the $U_{NOx}R$ can be used as a biomarker for disease state, we determined the $U_{NOx}R$ values in urine samples of the *LEW.1AR1-iddm* rat as a model of human T1DM.

Diabetic and control rats excreted similar amounts of protein in the urine (28±8 vs. 19±3 µg/mg creatinine). Diabetic rats excreted almost two times higher amounts of nitrate than control rats (139±24 vs. 73.8±8.4 µmol/mmol creatinine, P=0.032) (Table 1). This

finding agrees with a similar observation in T1DM humans (O´Byrne et al. 2000) and suggests enhanced NO synthesis in the diabetic animals. As urinary nitrate does not specifically reflect the activity of an individual NOS isoform, the elevated nitrate excretion seen in the diabetic rats of the present study and in patients with T1DM (O´Byrne et al. 2000) may reflect the activity of the inducible (iNOS) as well as eNOS and neuronal NOS (nNOS). Urinary nitrite excretion was almost four times higher in the diabetic rats compared to the healthy control animals (0.87±0.32 vs. 0.24±0.03 µmol/mmol creatinine, P=0.012). The $U_{NOx}R$ was significantly lower in the diabetic rats compared to the control animals (198±26 vs. 308±10, P=0.0079, Mann-Whitney test; Fig. 6A). These observations suggest that renal reabsorption of nitrite is impaired in the T1DM situation and leads to a higher excretion of nitrite. We found a very tight correlation (r=1.00; P=0.02) between urinary nitrite and nitrate concentrations in the diabetic rats indicating a coupled nitrite and nitrate renal excretion. There was a borderline correlation (r=0.87; P=0.08) between urinary nitrite and nitrate in the non-diabetic rats. Diabetic rats excreted statistically insignificantly higher amounts of the endogenous NOS inhibitor ADMA than controls (3.96±1.62 vs. 0.70±0.10 µmol/mmol, P=0.42; Fig. 6B).

With respect to the urinary excretion of the marker of lipid peroxidation 15(*S*)-8-*iso*-$PGF_{2\alpha}$, there was no significant difference between diabetic and control rats (21.5±3.93 vs. 18.4±2.21 nmol/mol creatinine, P=0.84; Fig. 6C), suggesting no elevated oxidative stress in the diabetic animals. No correlation was observed between urinary nitrite and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in the diabetic (r=0.70; P=0.23) and control (r=0.87; P=0.08) rats. Urinary nitrate and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ also did not correlate in the diabetic (r=0.70; P=0.23) and control (r=0.60; P=0.35) rats. There was no correlation between urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and $U_{NOx}R$ in the diabetic (r=-0.60; P=0.35) and control (r=-0.90; P=0.08) rats. $U_{NOx}R$ did not correlate with urinary ADMA in the diabetic or in the non-diabetic rats (data not shown).

In summary, renal CA-dependent nitrite reabsorption is decreased in the human T1DM *LEW.1AR1-iddm* rat model, presumably by mechanisms independent of oxidative stress.

$U_{NOx}R$ in dyslipidemic *APOE*3-Leiden.CETP* mice

The *APOE*3-Leiden.CETP* mice are a unique model for human-like lipoprotein metabolism, which shows a similar response to lipid-lowering drugs including statins as in humans both with respect to changes in magnitude and dosage of the drugs. In dyslipidemic *APOE*3-Leiden.CETP* mice treated with resveratrol and/or atorvastatin, only a combination of resveratrol and atorvastatin was found to increase slightly $U_{NOx}R$ (Fig. 7A), with no changes in the urinary concentrations of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (Berbée et al. 2013). Thus, the combination of resveratrol and atorvastatin is likely to improve the CA-dependent reabsorption of nitrite.

Of the antiretroviral protease inhibitor drugs tested, only ritonavir at 60 mg/kg bodyweight per day increased the $U_{NOx}R$ values in *APOE*3-Leiden.CETP* mice as compared to placebo (mean±SEM) from 194±52 to 463±60 (P=0.0056); a lower dose of ritonavir (10 mg/kg bodyweight per day) in combination with lopinavir had no effect (Fig. 7B). These findings suggest that ritonavir and/or its metabolites may impair renal CA-dependent nitrite reabsorption. Our results are in line with data from the literature that point to an inhibition of CA activity by ritonavir (Denissen et al. 1997; Supuran 2008; Haque et al. 2012).

Stable-isotope dilution techniques – the $U^{15}_{NOx}R$

In studies on renal CA-dependent nitrite reabsorption (Tsikas et al. 2010b; Chobanyan-Jürgens et al. 2012a) and CA-dependent formation of *S*-nitrosothiols (Zinke et al. 2015), the use of stable-isotope labelled nitrate and nitrite, notably [^{15}N]nitrate and [^{15}N]nitrite, is safe, provides valuable information and is therefore highly recommended in experimental and clinical studies. Urinary [^{15}N]nitrate and [^{15}N]nitrite may derive from ingested authentic inorganic [^{15}N]nitrite salts (Fig. 8A; Fig. S3). [^{15}N]Nitrate and [^{15}N]nitrite may also derive from [^{15}N]NO produced from ingested L-[*guanidine*-$^{15}N_2$]-arginine by NOS (Kayacelebi et al. 2015) (Fig. 8B). Finally, [^{15}N]nitrate and [^{15}N]nitrite may also derive from organic nitrates (Fig. 1) labelled with ^{15}N in their nitrate groups, such as [^{15}N]PETN, [^{15}N]ISDN or [^{15}N]GTN (glycerol trinitrate). Simultaneous measurement of [^{15}N]nitrate and [^{15}N]nitrite in blood and urine samples from such studies by GC-MS (Tsikas et al. 2010b), and calculation of the

$U^{15}_{NOx}R$ value generated significant information about renal CA-dependent nitrite reabsorption. $U^{15}_{NOx}R$ is simply calculated by dividing the peak area ratio (PAR) of the mass-to-charge (*m/z*) ratio of 63 ($^{15}NO_3^-$) to 62 ($^{14}NO_3^-$) to the PAR of *m/z* of 47 ($^{15}NO_2^-$) to 46 ($^{14}NO_2^-$) which corresponds to the ratio of the molar ratios [^{15}N]nitrate/[^{14}N]nitrate and [^{15}N]nitrite/[^{14}N]nitrite (Fig. S3). Thus, $U^{15}_{NOx}R$ is not entirely comparable with $U_{NOx}R$. Due to the natural abundance of 0.36% of the isotope ^{15}N of the element N, the molar ratio of [^{15}N]nitrate/[^{14}N]nitrate is almost equal with the molar ratio of [^{15}N]nitrite/[^{14}N]nitrite. It results from this that the value of $U^{15}_{NOx}R$ is practically equal to 1 in urine samples at baseline, that means, prior to ingestion of [^{15}N]nitrate, [^{15}N]nitrite or ^{15}N-labelled compounds such as L-[*guanidine*-$^{15}N_2$]-arginine that may serve as a source for [^{15}N]nitrate and/or [^{15}N]nitrite.

Upon ingestion of ^{15}N-labelled species such as the sodium salt of [^{15}N]nitrite, $U^{15}_{NOx}R$ may reach values above or below of 1. Fig. 8A shows that drinking of sodium[^{15}N]nitrite diluted in water by a healthy subject resulted in a gradual increase of the $U^{15}_{NOx}R$ value indicating a lower excretion rate of [^{15}N]nitrite in the urine compared to [^{15}N]nitrate which is produced from ingested [^{15}N]nitrite (see also Fig. S3). However, when [^{15}N]nitrite is ingested a few minutes after the ingestion of acetazolamide, the $U^{15}_{NOx}R$ value decreases suddenly to reach values below 1. This indicates that the excretion of [^{15}N]nitrite into the urine increased drastically compared to the urinary excretion of [^{15}N]nitrate. [^{15}N]Nitrate is rapidly formed from [^{15}N]nitrite oxidation in erythrocytes by oxyhemoglobin. Subsequently, the $U^{15}_{NOx}R$ value reaches its baseline value already 2 h after the second ingestion of sodium [^{15}N]nitrite.

In the two mice which drank for four days L-[*guanidine*-$^{15}N_2$]-arginine diluted in tap water, the $U^{15}_{NOx}R$ value increased from the baseline value of 1 to a plateau of a value around 2 after two days (Fig. 8B). This finding suggests that ingested L-[*guanidine*-$^{15}N_2$]-arginine was absorbed and partly oxidized to ^{15}NO. Subsequently, ^{15}NO was oxidized to [^{15}N]nitrate and [^{15}N]nitrite which were then excreted in the urine. Obviously, it takes about two days until a steady state is reached between L-[*guanidine*-$^{15}N_2$]-arginine absorption, NOS-catalyzed conversion to ^{15}NO and renal excretion of its metabolites [^{15}N]nitrate and

[^{15}N]nitrite. The increase of the $U^{15}_{NOx}R$ value from 1 to 2 suggests that the molar ratio of [^{15}N]nitrate and [^{15}N]nitrite formed from ^{15}NO produced by NOS from synthetic L-[*guanidine*-$^{15}N_2$]-arginine differs from the molar ratio of [^{15}N]nitrate and [^{15}N]nitrite obtained from ^{15}NO produced by NOS from endogenous L-[*guanidine*-$^{15}N_2$]-arginine, and from other sources (see Fig. 1).

Discussion

One of the major tasks of the renal proximal tubule is to secrete protons into the tubule lumen, thereby reabsorbing about 80% of the filtered bicarbonate (for review see Boron 2006). CAII and CAIV, sodium-proton exchanger 3 (NHE3), several aquaporins (AQP) and anion exchangers (AE) are parts of the renal proximal tubule arsenal that regulates the acid-base transport (Boron 2006). In humans, we found that renal CA isoforms are involved in the reabsorption of inorganic nitrite (NO_2^-) and to a lesser extent of inorganic nitrate (ONO_2^-) (Tsikas and Chobanyan-Jürgens 2010; Tsikas et al. 2010; Chobanyan-Jürgens et al. 2012a). Purified bovine erythrocytic CAII has been reported to generate NO from nitrite in the presence of the CA inhibitor dorzolamide (Aamand et al. 2009). The underlying mechanisms of these potentially novel functions of the CA family are not yet known. Recent findings from our group indicate that bovine and recombinant human CAII and CAIV acidify NO_2^- to nitrous acid (HONO) which is chemically reactive and undergoes further reactions (Zinke et al. 2015) (Fig. 1). They include *S*-nitrosylation of cysteinyl thiols (RCysSH) which leads to formation of *S*-nitrosothiols such as GSNO and CysSNO (Hanff et al. 2015). *S*-Nitrosothiols exert NO-related bioactivity via cGMP-dependent and cGMP-independent mechanisms (Tsikas et al. 1999). Furthermore, phosphorylation of CA, another posttranslational modification, has been shown to modulate its intrinsic carbonic anhydrase activity (Carrie and Gilmour 2015).

Nitrite may directly derive from NO (via autoxidation) or from nitrate (including nitrate produced from NO and nitrite oxidized by oxyhemoglobin in erythrocytes) by the

action of nitrate reductases in the mouth and gut flora (Fig. 1). In vitro, we found that nitrite can react chemically with CO_2 to form most likely nitritocarbonate ($O=N-O-COO_2^-$) and that this reaction can be modulated by bovine erythrocytic CAII (Chobanyan-Jürgens et al. 2012; Tsikas et al. 2016). Inorganic nitrite and nitrate are mainly eliminated by the kidneys. In healthy adults, the creatinine-corrected excretion rates of nitrite and nitrate are in the range 0.08 – 0.30 μmol/mmol creatinine and 90 – 110 μmol/mmol creatinine, respectively, i.e., the nitrate excretion is more than two orders of magnitude higher than that of nitrite (Tsikas 2008). In humans, oral intake of the CA inhibitor acetazolamide resulted in highly enhanced excretion of endogenous nitrite and of orally administered [^{15}N]nitrite (Tsikas and Chobanyan-Jürgens 2010; Tsikas et al. 2010; Chobanyan-Jürgens et al. 2012a), suggesting that the reabsorption of nitrite from the primary urine is mediated, at least in part, by renal CA isoforms. The molar ratio of urinary nitrate to urinary nitrite, i.e., $U_{NOx}R$, describes the urinary excretion of nitrite relative to nitrate (Fig. 1). Thus, we hypothesized that $U_{NOx}R$ may be a useful measure of renal CA-dependent reabsorption of urinary nitrite in humans. $U_{NOx}R$ considers the $ONO_2^-/NO_2^-/NO$ cycle (Weitzberg and Lundberg 2013) and may therefore provide important quantitative information about the size of the NO reservoir/bioavailability in the circulation (Fig. 1). We assumed that higher $U_{NOx}R$ values would indicate a more abundant $ONO_2^-/NO_2^-/NO$ cycle and thus a healthier organism than lower $U_{NOx}R$ values. We were therefore interested in learning more about this potentially useful biomarker in different conditions. In this work, we present novel results and undertake a first evaluation of the utility of $U_{NOx}R$ as a measure of renal CA-dependent reabsorption of nitrite in experimental and human studies by performing a meta-analysis of results previously reported by our and other groups (Table 1).

Autoimmune and inflammatory diseases

In healthy adults, average $U_{NOx}R$ reaches values ranging between about 400 and 1600 (Table 1). Autoimmunity and inflammation characterize rheumatic diseases, T1DM and to a lesser extent also T2DM. The several times lower $U_{NOx}R$ values we measured in adult patients suffering from T2DM or chronic rheumatic diseases, including rheumatoid arthritis,

spondyloarthritis and vasculitis (Table 1), are indicative of an impaired renal CA-dependent reabsorption of nitrite (Fig. 1). The finding that the $U_{NOx}R$ was independent of age, disease duration and markers of inflammation in the T2DM patients of the DALI study suggests that impairment of this kind of CA activity may manifest itself early in the disease and may not further exacerbate in elderly patients with age, disease duration and inflammation degree. Compared to healthy elderly subjects, elderly patients with PAOD or CAD had similar $U_{NOx}R$ values (Table 1), which were moreover comparable with those measured in ten obese but otherwise healthy men (Table 1).

The finding that T1DM rats have lower $U_{NOx}R$ values than healthy non-diabetic rats greatly supports the idea that T1DM rats suffer from impaired renal CA-mediated reabsorption of urinary nitrite. Because our rat model shares major characteristics with human T1DM (Jörns et al. 2014), T1DM patients may also suffer from diminished NO bioactivity as a result of impaired renal CA-dependent reabsorption of nitrite. This is of particular importance because nitrite in the circulation and in tissues is considered a major NO reservoir (Gladwin et al. 2006) (Fig. 1).

As cardiovascular complications are rare in early stage T1DM patients, we hypothesise that the renal reabsorption of nitrite is intact or only weakly impaired in T1DM. Unfortunately, we have no data about $U_{NOx}R$ in adults suffering from T1DM. It should be pointed out that the L-Arg/NO pathway differs greatly in childhood and adulthood, with differences also including circulating and excretory nitrate and nitrite (Lücke et al. 2007). Therefore, U_{NOxR} values measured in childhood and adulthood are not easy to compare. It is worth mentioning that in 102 T1DM children the $U_{NOx}R$ values (mean±SD) of 1192±592 are even higher than those measured in healthy non-diabetic children (Carmann et al. 2015) (Table 1). However, in children suffering from Duchenne muscular dystrophy (DMD), the $U_{NOx}R$ values were significantly lower compared to healthy children and those with T1DM (Table 1). Interestingly, newly diagnosed untreated T1DM children and medicated T1DM children were found to have comparable $U_{NOx}R$ values. However, insulin-treatment has halved the urinary excretion of both, nitrite and nitrate, to a comparable degree.

Atorvastatin administration at 10 mg/d or 80 mg/d for 30 weeks was found not to alter synthesis of NO, prostacyclin or thromboxane, and not to decrease oxidative stress in the adult T2DM patients of the DALI study (Tsikas et al. 2015). These observations are in line with the absence of an appreciable change in $U_{NOx}R$ upon atorvastatin treatment both in the DALI study and in the *APOE*3-Leiden.CETP* mice. In this model, only a combination of resveratrol and atorvastatin slightly increased $U_{NOx}R$, with no changes in the urinary concentrations of nitrite, nitrate and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (Tsikas et al. 2015), a widely used biomarker of oxidative stress (Tsikas 2017). In T2DM, the correlation between $U_{NOx}R$ with CRP became significant after 30 weeks of atorvastatin administration $U_{NOx}R$ (r=0.318, P=0.0001). This may be due to the atorvastatin-mediated reduction of the CRP levels in the DALI study (van der Ree et al. 2003).

In the *APOE*3-Leiden.CETP* mice, of the orally administered HIV-1 protease inhibitors tested, i.e., ritonavir, lopinavir and atazanavir, only ritonavir lowered $U_{NOx}R$. This observation suggests that ritonavir itself and/or its metabolites might have inhibited CA-mediated reabsorption of nitrite in the kidneys of the animals, thus leading to a higher loss of NO bioavailability in the form of elevated urinary excretion of nitrite. The finding that the lopinavir/ritonavir combination did not lower significantly the $U_{NOx}R$ value is likely to be due to a lacking effect of lopinavir on renal CA-mediated reabsorption of nitrite in combination with a smaller ritonavir effect because of its lower dose used in the combination with lopinavir compared with ritonavir alone (10 mg/kg/d vs. 60 mg/kg/d). This finding is supported by observations indicating that many drugs, including protease inhibitors such as ritonavir, cause proximal renal tubular acidosis, most likely by interacting with renal CA isoforms in the proximal tubule (Liu et al. 2012). An explanation for the inhibitory effect of ritonavir and the lacking effects of lopinavir and atazanavir on nitrite reabsorption by renal CA isoforms is likely to be due to the thiazole moiety of ritonavir, which is absent in lopinavir and atazanavir. In humans, ritonavir is metabolized to numerous more polar metabolites of which many contain the non-substituted thiazole moiety (Denissen et al. 1997). Three of the identified ritonavir metabolites are formed by decarbamoylation of ritonavir. The thiazolyl moiety released by this biotransformation is expected to be

chemically unstable and is likely to lose CO_2 thereby forming 4-(hydroxymethyl)-1,3-thiazol. The latter is structurally related to the CA inhibitor acetazolamide and other biologically active drugs, and is bearing furthermore a chemically reactive thiocyanate group like the experimental CA inhibitor DIDS (Tsikas et al. 2013).

Ethnic differences with regard to $U_{NOx}R$

Hypertension is a particularly common finding in black populations. Hypertension occurs at younger ages and results in a higher incidence of cardiovascular disease and mortality in blacks, yet the reasons are incompletely understood (Schutte et al. 2017). The lower $U_{NOx}R$ values found in the black boys of the present study (Table 1) could be an indication of a lower reabsorption rate of nitrite compared to the white boys, presumably due to genetic differences in renal CA isoforms and anion transporters.

$U_{NOx}R$ as an indicator and measure of tolerance development to organic nitrates?

An interesting finding of our study is that at therapeutically relevant and equivalent oral dosages, the organic nitrates PETN and ISDN caused a decrease in the $U_{NOx}R$ values already one day after starting drug administration, whereas the decrease was more pronounced for ISDN. By definition, high $U_{NOx}R$ values indicate efficient CA-dependent reabsorption of urinary nitrite, while low $U_{NOx}R$ values indicate impaired CA-dependent nitrite reabsorption thus resulting loss of NO bioactivity (Fig. 1). Based on these assumptions, our findings concerning the urinary excretion of nitrite and nitrate upon oral intake of the organic nitrates by healthy young adults would indicate that intake of ISDN causes a stronger loss of NO bioactivity compared to PETN. Such an interpretation would be in line with the currently prevailing and increasingly supported idea that ISDN but not PETN induces tolerance and endothelial dysfunction (Thum et al. 2011; Münzel et al. 2014). Previously, we demonstrated that in healthy young men and at therapeutically relevant doses neither ISDN nor PETN induce oxidative stress (Keimer et al. 2003), which is considered a major factor in endothelial dysfunction because it decreases NO bioavailability through NO oxidation to nitrite, nitrate and peroxynitrite. It is interesting to note that chronic oral

ingestion of inorganic nitrate, i.e., drinking of a $NaNO_3$ solution in tap water, did not alter the $U_{NOx}R$ values in young healthy men (Hanff et al. 2017). Men (n=9) who ingested $NaNO_3$ (0.1 mmol/kg/d) had even higher $U_{NOx}R$ values than men (n=8) who ingested placebo (NaCl, 0.1 mmol/kg/d): 1709±355 vs. 369±77, without concomitant changes in plasma creatinine concentration (Hanff et al. 2017). The incompletely understood mechanisms underlying the still enigmatic phenomenon of tolerance development to organic nitrates (Mayer and Beretta 2008) and the potential role of renal CA isoforms remain to be further investigated, and $U_{NOx}R$ could be a useful parameter in forthcoming studies.

Mechanistic considerations

The mechanisms by which nitrite and nitrate are excreted into and reabsorbed from the primary urine in humans are incompletely understood. Our studies, especially those involving the use of acetazolamide, a strong clinically used CA inhibitor, indicate that one important mechanism is likely to be based on renal CA isoforms (Fig. 1). In addition to renal CA, anion transport systems may also regulate the excretion and the reabsorption of the anions nitrite and nitrate in the kidney, presumably in parallel with the CA-dependent bicarbonate transport. It is noteworthy that inorganic nitrite (NO_2^-), but not inorganic nitrate (NO_3^-), reacts chemically with CO_2, i.e., the substrate of CA isoforms including CAIV (Tsikas et al. 2013, 2016). This specific reaction could make nitrite reabsorption in the nephron even more accessible to variations in CA activity and expression in the kidneys. The underlying mechanism of the elevated nitrite excretion in diabetes and chronic rheumatic diseases in adults is likely to involve impaired reabsorption of nitrite in the proximal tubule as the result of altered activity and/or expression of the membrane-bound CAIV and the cytosolic CAII in the nephron. Literature reports (Liu et al. 2012; Gambhir et al. 2007) are supportive of the involvement of renal (and erythrocytic) CA isoforms in diabetes and other autoimmune diseases.

A considerable fraction of circulating nitrite and nitrate originates from sources other than the L-Arg/NO pathway (Fig. 1). A major contributor is nutrition (Weitzberg and

Lundberg 2013). Our studies indicate that the $U_{NOx}R$ may vary considerably (by 50 to 60%) in healthy non-medicated humans. One important factor for the $U_{NOx}R$ variability seems to be the pH value of the urine, which physiologically varies between about 4.5 and 8.5 over the day in healthy humans (Bilobrov et al. 1990; Murayama et al. 2001). Inhibition of renal CA activity by drugs such as acetazolamide will acutely and strongly decrease the $U_{NOx}R$ over several hours. Other widely used drugs such as NAC (Tsikas et al. 2014) and paracetamol (Trettin et al. 2014) are weak CA inhibitors and are not expected to decrease strongly the $U_{NOx}R$ value when used at therapeutically relevant doses. Yet, individuals may respond differently to these and possibly to other drugs.

In male white mice (18-20 g bodyweight), intraperitoneal injection of $NaHCO_3$ (2000 mg/kg) was found to reduce methemoglobinemia previously induced by intraperitoneal injection of $NaNO_2$ (150 mg/kg) and to prolong the survival time by improving vital biochemical pathways including the antioxidative defense system in erythrocytes (Shugaleĭ et al. 1994). This group assumed that the protective effect of administered bicarbonate was due to its reaction with hydrogen peroxide, superoxide and hydroxyl radicals (Shugaleĭ et al. 1994). Although such reactions cannot be excluded, we think that the main effects were due to greatly enhanced renal elimination of nitrite rather than of $NaNO_2$-derived nitrate. Obviously, alkalization of the urine by pharmacological bicarbonate resembles the effect of the clinical CA inhibitor acetazolamide on nitrite reabsorption (see Fig. 2). Unfortunately, measurements of nitrite, nitrate, bicarbonate and pH in blood and urine samples of the mice intoxicated by $NaNO_2$ had not been reported (Shugaleĭ et al. 1994).

Recent studies indicate that certain CA isoforms are responsible for the chemical activation of nitrite to strongly nitrosating NO-species (Aamand et al. 2009; Zinke et al. 2016; Hanff et al. 2016). Protons provided by CA at neutral pH are likely to protonate nitrite to nitrous acid (HONO, pK_a 3.4) which may also form its anhydride (N_2O_3) (Fig. 1). HONO and N_2O_3 are potent nitrosating species. Thus, bovine and human CAII and CIV were found to nitrosate thiols on their sulfur atom (*S*-nitrosylation) and tyrosine on one of its aromatic C atoms (*C*-nitrosylation) (Zinke et al. 2016, Hanff et al. 2016). Physiological *S*-nitrosothiols such as *S*-nitrosohemoglobin (HbCysSNO), GSNO and CysSNO can exert biological activity

by mechanisms depending on cGMP or independent of cGMP (Tsikas et al. 1999). Well-known biological activity exerted by HbCysSNO, GSNO and CysSNO includes vasodilation and inhibition of platelet aggregation (Tsikas 2008) (Fig. 1). Because of the lacking specificity of HONO and N_2O_3 towards substrates, these species may also nitrosate amines on their amine groups. It is therefore reasonable to assume that CA may also catalyze the *N*-nitrosylation of particular amines and amino acids (Fig. 1). As an example, sarcosine (*N*-methylglycine) is subject to *N*-nitrosation to form *N*-nitrososarcosine. This nitrosamine is chemically highly reactive and a potent alkylating species towards biomolecules such as guanine, and as such has a mutagenic and cancerogenic potential (de Vogel et al. 2014; Krishnamurthy et al. 2006, 2013). Thus, CA is a versatile enzyme family which possesses many additional activities and functions beyond its inherent carbonic anhydrase activity and pH regulation in various biological systems.

Possible limitations for the clinical use of $U_{NOx}R$ as a measure of renal CA-dependent reabsorption of nitrite

Very low $U_{NOx}R$ values may arise from high (lower µM-range) urinary nitrite concentrations due to bacteriuria (Chao et al. 2016) and/or improper collection and storage conditions of urine samples. It is noteworthy that in urine nitrate exists in a very high molar excess over nitrite, so that reduction of even a very small fraction of nitrate to nitrite by bacterial nitrate reductases may considerably decrease the $U_{NOx}R$ value. In the studies reported in the present work such unfavourable conditions were avoided. The highest urinary nitrite concentrations and the lowest $U_{NOx}R$ values were measured in the T2DM patients of the DALI study. The prevalence of (asymptomatic) bacteriuria in T1DM and T2DM inpatients and outpatients is about two times higher than in non-diabetic persons and may reach values ranging between 2 and 14% of the whole cohort (Keane et al. 1988; Ishay et al. 2006; Matteucci et al. 2007; Renko et al. 2011). Complete avoidance of bacteriuria in subjects participating in clinical studies is therefore almost impossible. Nevertheless, bacteriuria must not always be associated with enhanced bacterial reduction of nitrate to nitrite. Bacterial colonization in the urinary tract may also induce iNOS expression in macrophages,

thus contributing large amounts of L-Arg/NO-derived nitrite independent of bacterial nitrate reductase activity. Recently, we discovered that CA isoforms are also expressed in human penile erectile tissue (Ückert et al. 2016). This may be of particular importance because many CA isoforms are associated with cancer (Takakura et al. 2012). For many decades inorganic nitrite from certain foods is considered as a key nitrosating species (Lewin et al. 2006). It remains to be investigated whether certain CA isoforms are also involved in the bioactivation of nitrite to form alkylating *N*-nitrosamines, i.e., beyond NO and RSNO formation (N-Nitrosamines (15 listings): N-Nitrososarcosine 2011). Sarcosine (*N*-methylglycine) is considered as a biomarker for prostate cancer (Sreekumar et al. 2009; Cheng et al. 2010; Cernei et al. 2013) (Fig. 1).

Several bacteria are known to express CA, and inhibition of bacterial CA can lead to debilitation and changes in the metabolism of the microorganisms (Capasso and Supuran 2017), presumably including the nitrate-to-nitrite conversion rate. For this and other reasons (Sütö et al. 1995; present study), $U_{NOx}R$ in experimental and clinical studies involving use of CA inhibitors is likely to be compromised.

In some of our human studies, urine from spontaneous micturition was freshly collected and immediately analyzed or was frozen aliquoted at -20 °C. In other human studies, in which urine had to be collected over the day (e.g., Keimer et al. 2003; Tsikas and Kinzel 2017), we took precautions to avoid both, loss of nitrite which takes place under acidic conditions and artefactual formation due to bacterial nitrate reductase activity. Use of control groups, standard operation procedures for nutrition (i.e., low nitrate/nitrite diet), medication (e.g., organic nitrates), overnight fasting, as well as for urine sampling and storage and use of preservatives, such as a combination of EDTA and 4-hydroxy-TEMPO or organic solvents, and use of highly specific methods for the accurate measurement of nitrite in the presence of huge amounts of nitrate are required for the reliable assessment of $U_{NOx}R$ in experimental and clinical studies. These issues are of particular importance in studies involving children, where urine collection often represents a pragmatic challenge.

Being small anions, both nitrate and nitrite can be transported by several different cellular mechanisms. Most of the reported studies have used nitrate and nitrite at very high

mM-concentrations. In pancreatic acinar cells, the transport of nitrite was found to be coupled to CO_2-bicarbonate transporters and to be affected by CA leading to cytosolic acidification (e.g., by 0.034 pH-units at 0.1 mM nitrite; Zhao et al. 1994). This group considered nitrous acid (HONO) and other higher nitrogen oxides as the acidifying species in a manner depending on CA. In a variety of mammalian cells, nitrate (used at mM concentrations) has been reported to be transported by NO_3^--H^+ cotransporters which were only marginally dependent upon CA (Chow et al. 1997). Anion exchangers (AE) including AE1, AE2, and AE3, the main chloride-bicarbonate co-transporters, have been shown to be coupled to CAII thus building a bicarbonate transport metabolon (Sterling et al. 2001). This interaction seems to be required for maximum bicarbonate transport. Eventually, aquaporin-6 (AQP-6) has been reported to serve as a channel for nitrate in mammalian cells (Ikeda et al. 2002). Obviously, nitrate and nitrite are transported by several different mechanisms which are in part coupled to the bicarbonate transport and thus to CA activity. The quantitative contribution of the individual transport mechanisms is currently unknown and remains to be determined in sophisticated studies. Our studies suggest that in humans the renal CAII and CAIV have considerable selectivity towards nitrite (Tsikas and Chobanyan-Jürgens 2010; Chobanyan-Jürgens et al. 2012a; Tsikas et al. 2014). In the far majority of our studies, urinary nitrate and nitrite correlated directly with each other (correlation coefficient range, 0.335 – 0.893). Without exception, creatinine-corrected excretion of nitrite correlated inversely with $U_{NOx}R$ (correlation coefficient range, 0.348 – 0.893), while the correlation of $U_{NOx}R$ with the creatinine-corrected excretion of nitrate was not uniform, albeit mostly positive. These studies suggest that the urinary nitrite excretion is a much stronger predominant term of $U_{NOx}R$ than urinary nitrate excretion. $U_{NOx}R$ may therefore be useful as a measure of renal CA-dependent nitrite reabsorption in health and disease. The reabsorption capacity of renal CA and other transport systems towards urinary nitrite is unknown. The results of our clinical study with PETN and ISDN suggest that the renal reabsorption capacity is limited. Daily intake of 240 mg (0.76 mmol) PETN or 90 mg (0.38 mmol) ISDN can considerably decrease the $U_{NOx}R$ value. Considering stoichiometric and quantitative conversion of the nitrate groups of PETN and ISDN to NO, it seems that

daily formation of 3.04 mmol NO from PETN and of 0.76 mmol NO from ISDN can decrease remarkably the $U_{NOx}R$ and thus the renal reabsorption capacity for nitrite. It remains to be demonstrated whether partly and/or entirely denitrated metabolites of PETN and ISDN are involved in the reduction of $U_{NOx}R$, while chronic ingestion of high amounts of inorganic nitrate does not decrease $U_{NOx}R$. On the other hand, inhibition of renal CA by acetazolamide seems to force the diuresis of nitrite for several hours in parallel to bicarbonate.

Summary

There is increasing evidence that the excretion of nitrite in the urine is coupled, at least in part, to the CA activity in the proximal tubule of the nephron. The molar ratio of urinary nitrate to urinary nitrite, i.e., the measure $U_{NOx}R$, may be a suitable measure of the CA-dependent reabsorption of nitrite in adults and children (Table 1, Fig. S4). Thus, $U_{NOx}R$ may be a useful measure of the NO bioavailability in the renal and cardiovascular systems in health, disease and pharmacotherapy. In PAOD patients, $U_{NOx}R$ is inversely correlated with blood pressure and suggests that elevated excretion or diminished reabsorption of nitrite may decisively determine NO-dependent biological activity, namely vasodilation. Of particular importance could be the use of $U_{NOx}R$ in identifying and quantitating tolerance to established and novel organic nitrates, to other so-called NO donors and to the currently increasingly used inorganic nitrate and nitrite in experimental and clinical studies. Accurate determination of $U_{NOx}R$, which seems to vary considerably in humans, requires use of proper pre-analytical precautions, notably avoidance/minimization of bacterial infection, and most importantly use of specific and sensitive analytical methods for urinary nitrite (upper nM- to lower µM-range) in the presence of high molar excess of nitrate (upper µM- to lower mM-range). The simultaneous GC-MS measurement of nitrite and nitrate in urine samples as pentafluorobenzyl derivatives (Tsikas 2000) is best suited for the study of nitrite-related CA activity in the urogenital, renal and cardiovascular systems. Of particular importance is the CA-dependent reabsorption of nitrite in the kidney and the bioactivation of nitrite to *S*-nitrosothiols, NO, nitrosoamines and nitrotyrosine in various types of cell including

erythrocytes and muscle cells. Acetazolamide may decrease systolic blood pressure and increase cardiac output, thereby increasing cerebral blood flow (Hauge et al. 1983). Acetazolamide can enhance nitrite-induced radial artery dilation (Omar et al. 2015). It can thus be expected that CA isoforms may exert different nitrite-related effects in the brain, in the cardiovascular and renal systems.

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Ethical statement

The studies reported in this work were approved by the local Ethics Committees and the Institutional Ethics Committees on Animal Care and Experimentation.

Conflict-of-interest disclosure

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

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Figure legends

Fig. 1. Sources of nitric oxide (NO), nitrite and nitrate and simplified proposed mechanisms for the bioactivation of inorganic nitrite. For more details see the text. Abbreviations: CA, Carbonic anhydrase; CysSH, L-cysteine; CysSNO, *S*-nitroso-L.cysteine; GSNO, *S*-nitroso glutathione; Hb, hemoglobin; HbFeNO, nitrosyl hemoglobin; HbCysSNO, *S*-nitroso hemoglobin; N_xO_y, higher nitrogen oxides; $U_{NOx}R$, urinary nitrate-to-nitrite molar ratio; XOR, xanthine oxidoreductase. Symbols: (+), activation; (-), inhibition.

Fig. 2. Mean urinary concentrations of nitrate, nitrite and bicarbonate (A), mean creatinine-corrected urinary excretion of nitrate (B) and nitrite (C), and mean $U_{NOx}R$ (D) in urine samples collected by seven healthy subjects before and after oral intake of the CA inhibitor acetazolamide (a 500-mg Diamox retard capsule, corresponding to 5 mg/kg body weight). This Figure was constructed by using the data reported in previous study (Tsikas and Chobanyan-Jürgens 2010) and the newly measuring nitrite and nitrate concentrations in the urine samples (see Fig. 5B in Tsikas and Chobanyan-Jürgens 2010). The vertical dotted line in (A) separates regimes with existing and missing correlations between the parameters.

Fig. 3. (A) $U_{NOx}R$ in 18 healthy previously non-medicated young men at baseline on day 1 and day 21. (B) Decadic logarithm of $U_{NOx}R$ in 18 healthy previously non-medicated young (DAY 0), after 1 day (DAY 1), and after 5 (DAY 5) days of oral intake of PETN or ISDN. This Figure was constructed by using the individual urinary nitrate and nitrite concentrations reported elsewhere (Keimer et al. 2003).

Fig. 4. Relationship between systolic or diastolic pressure and $U_{NOx}R$ in 37 elderly patients suffering from peripheral artery occlusive disease (PAOD). The anthropometric and clinical characteristics of the PAOD patients have been reported in previous study (Schneider et al. 2015). The Figure was prepared using the baseline data of this study.

Fig. 5. $U_{NOx}R$ in healthy non-medicated men (n=25) and women (n=16) (A), and relationship between $U_{NOx}R$ and urinary pH (B) or age (C) in the whole group. This Figure was constructed by using the individual nitrate and nitrite concentrations reported elsewhere (Pham et al. 2009).

Fig. 6. $U_{NOx}R$ (A), ADMA (B) and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (C) in healthy non-diabetic rats (n=5) and in T1DM rats (n=5). Data are presented as mean± SEM.

Fig. 7. (A) $U_{NOx}R$ in untreated (Control) and *APOE*3-Leiden.CETP* mice fed with resveratrol (Res), atorvastatin (Ator), or with resveratrol plus atorvastatin (Res+Ator). This Figure was constructed using newly determined urinary nitrate and nitrite concentrations of a previously reported study (Berbeée et al. 2013). (B) $U_{NOx}R$ in untreated (Placebo) and *APOE*3-Leiden.CETP* mice treated with ritonavir, lopinavir+ritonavir or atazanavir (n=9 per group). Data are presented as mean±SEM.

Fig. 8. (A) Urinary [^{15}N]nitrate-to-[^{15}N]nitrite molar ratio $U^{15}_{NOx}R$ in a healthy female volunteer (age, 25 years) who drank two times [^{15}N]nitrite sodium dissolved in drinking water (0.31 µmol/kg each) and took once acetazolamide (5.4 mg/kg bodyweight) at the time points indicated by arrows. The first [^{15}N]nitrite occurred at the time point "0". This Figure was constructed using data of a previously reported study (Tsikas et al. 2010b). Note that the $U^{15}_{NOx}R$ value is calculated by dividing the ratio [^{15}N]nitrate/[^{14}N]nitrate by the ratio [^{15}N]nitrite/[^{14}N]nitrite. GC-MS chromatograms from urine analyses collected before and after [^{15}N]nitrite sodium intake are shown in the Supplement (Fig. S3). (B) $U^{15}_{NOx}R$ in two mice which drank L-[*guanidine*-$^{15}N_2$]-arginine-containing drinking water (100 µg/mL, 568 µM) for four days. [^{15}N]nitrate, [^{15}N]nitrite, [^{14}N]nitrate and [^{14}N]nitrite were measured simultaneously by GC-MS (Tsikas 2000). This Figure was constructed using data of a previously reported study (Tsikas 2004). Note that the $U^{15}_{NOx}R$ value is calculated by dividing the ratio [^{15}N]nitrate/[^{14}N]nitrate by the ratio [^{15}N]nitrite/[^{14}N]nitrite. Due to the natural abundance of 0.36% of the isotope ^{15}N of the element N, the ratios [^{15}N]nitrate/[^{14}N]nitrate and [^{15}N]nitrite/[^{14}N]nitrite are practically equal and the $U^{15}_{NOx}R$ value is close to 1 in urine samples collected prior to drink synthetic L-[*guanidine*-$^{15}N_2$]-arginine-containing tap water.

Table 1 Summary of baseline urinary nitrate, nitrite and $U_{NOx}R$ values reported in the literature and in the present work for healthy and diseased humans, rats and mice, and calculated from reported urinary excretion rates of nitrate and nitrite

Health state	Age (years)	$[NO_3^-]$ (μmol/mmol creatinine)	$[NO_2^-]$ (μmol/mmol creatinine)	$U_{NOx}R$ (—)	Reference
Adults [a]					
Healthy men (*n*=18)	26.4 ± 2.7	97.6 ± 13.2	0.075 ± 0.009	1570 ± 233	Keimer et al. (2003)
Healthy men (*n*=15)	24.2 ± 2.5	90.5 ± 15.6	0.29 ± 0.06	406 ± 61	Fahlbusch et al. (2004)
Healthy (16 f/25 m)	47 [26 – 82]	106 ± 12	0.19 ± 0.02	660 ± 72	Pham et al. (2009)
Hypogonadal men (*n*=8)	49 ± 8.9	74 [54-91]	0.56 [0.37-0.79]	123 ± 33	Tsikas & Kinzel (2017)
Rheumatic (18 f/10 m)	55 [23 – 82]	105 ± 13	1.10 ± 0.30	187 ± 22	Pham et al. (2009)
Transplantation (2 f/7 m)	45.7 ± 14.7	89 ± 80	0.55 ± 0.92	689 [100-1056] [b]	Becker et al. (2009)
		125 ± 148	1.43 ± 1.00	55.6 [34-133] [c]	
		147 ± 123	2.76 ± 2.13	38.4 [24-125] [d]	
CAD (12 f/48 m)	62	87.0 [80-125] (PLA)	0.19 [0.13-0.58] (PLA)	408 [236-736] (*n*=168)	Schneider et al. (2015)
		78.0 [72-117] (ARG)	0.20 [0.16-0.57] (PLA)		
PAOD (9 f/31 m)	68 ± 8	68.1 [55-105]	0.18 [0.11-0.30]	400 [295-579]	Schneider et al. (2015)
Obese (*n*=10 m; BMI>25)	21 – 50	76.8 [48-98]	0.22 [0.16-0.35]	346 [245-479]	Schneider et al. (2015)
T2DM (*n*=61-64)	45 - 75	162 ± 31	2.28 ± 0.26	68 ± 4	Tsikas et al. (2015)
Healthy subjects (*n*=241)	41 ± 15	1890 ± 1710 μM	0.89 ± 0.86 μM	1493 ± 1852	Chao et al. (2016)
UTI patients (*n*=73) [e]	73 ± 14	360 ± 320 μM	15.2 ± 65 μM	23.7	Chao et al. (2016)
Acromegaly (22 f/20 m)	46.1 ± 1.6	68.8 ± 69.6	0.13 ± 0.01	523	Present study
Neonates, children and adolescents					
Preterm neonates (*n*=76)	23+6 to 36+1 weeks	283 ± 85	4.3 ± 10.7	210 ± 14	Buck et al. (2017)
L-Arginine test (*n*=7) (t=0 h)	10 ± 2	132 [70-207]	0.34 [0.19-0.85]	359 [245-403]	Schneider et al. (2015)

L-Arginine test (*n*=7) (t=2 h)		149 [105-190]	0.69 [0.44-0.74]	220 [166-256]	Schneider et al. (2015)
Healthy (*n*=45)	11.1 ± 4.9	187 ± 335	0.46 ± 0.95	730 ± 591	Hörster et al. (2015)
Healthy (*n*=95)	11.3 [8-13.3]	117 [85-164]	0.22 [0.13-0.37]	540 [324-962]	Carmann et al. (2015)
DMD (*n*=54)	11.9 ± 4.8	230 ± 126	0.97 ± 1.10	424 ± 353	Hörster et al. (2015)
T1DM untreated (*n*=10)	8.8 [4.4-11.2]	176 [149-390]	0.18 [0.11-0.33]	1341 [1117-1615]	Carmann et al. (2015)
T1DM treated (*n*=92)	12.5 [10.5-15.4]	101 [72-158]	0.09 [0.06-0.17]	1173 [738-1481]	Carmann et al. (2015)
Hypercholesterolemia (*n*=64)	11.5 ± 3.5	125 [99-164]	0.19 [0.10-0.48]	608 [211-1152]	Chobanyan-J. et al. (2012)
Normocholesterolemia (*n*=54)	11.9 ± 4.6	118 [78-154]	0.23 [0.15-0.45]	499 [274-890]	Chobanyan-J. et al. (2012)
Phenylketonuria (*n*=52)	12 ± 7	218 ± 218	1.7 ± 1.7	128 (ratio of the means)	Kanzelmeyer et al. (2012)
Healthy controls (*n*=46)	12 ± 7	280 ± 439	0.7 ± 1.2	400 (ratio of the means)	Kanzelmeyer et al. (2012)
FSGS (*n*=9)	11 ± 4	262 ± 419	3 ± 5	87 (ratio pgf the means)	Lücke et al. (2008)
Non-FSGS (*n*=11)	4 ± 3	174 ± 204	1.3 ± 1.5	134 (ratio of the means)	Lücke et al. (2008)
Healthy controls (*n*=9)	11 ± 5	421 ± 712	1.3 ± 2	324 (ratio of the means)	Lücke et al. (2008)
Healthy black boys (*n*=39)	7.3 ± 0.7	67 [60.5-92.8] [f]	0.26 [0.18-0.46]	483 ± 314 [g]	Present study
Healthy white boys (*n*=41)	7.3 ± 0.8	94 [81.7-134]	0.25 [0.21-0.31]	314 ± 155	Present study

Animals

LEW.1AR1-iddm rat (healthy, *n*=5)		73.8 ± 8.4	0.24 ± 0.03	308 ± 10	Present study
LEW.1AR1-iddm rat (T1DM, *n*=5)		139 ± 24	0.87 ± 0.32	198 ± 26	Present study
*APOE*3-Leiden.CETP* mice (*n*=7)		88.4 [76-159]	0.80 [0.50-1.04]	155 [124-180]	Present study
*APOE*3-Leiden.CETP* mice (*n*=9)		474 [371-613]	1.10 [0.75-1.45]	458 [318-557]	Present study

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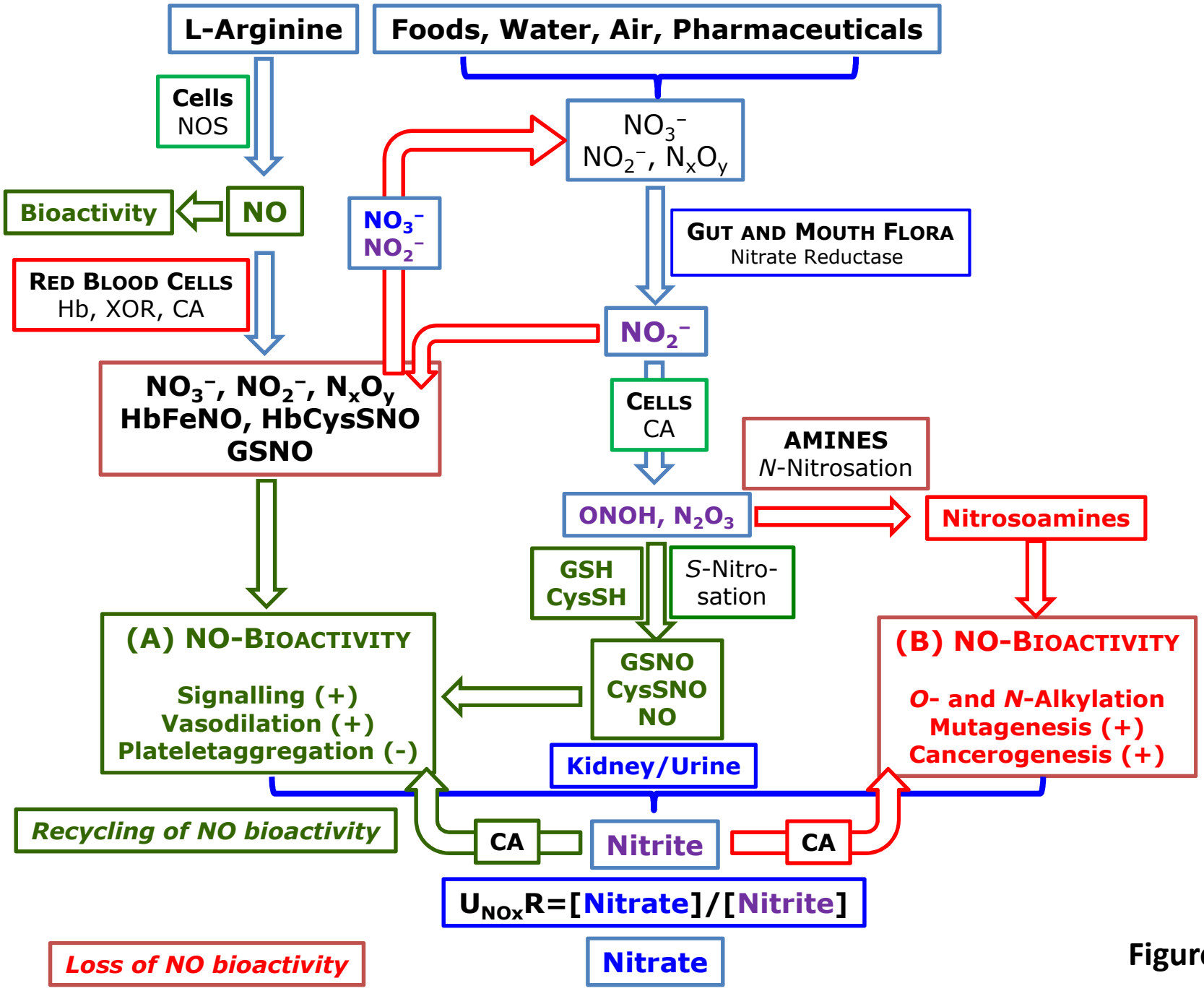
[a] Abbreviations: m, male; f, female.
[b] Pre-operatively (baseline); [c] post-operatively, 40-60 min: [d] post-operatively: 60-240 min.
[e] Hospitalized patients with urinary tract infections (UTI).
[f] *P*=0.0065, Mann-Whitney test, black vs. white boys for $U_{NOx}R$.
[g] *P*=0.0007, Mann-Whitney test, black vs. white boys for urinary nitrate.
ARG, L-arginine group; PLA, placebo group


L-Arginine
Foods, Water, Air, Pharmaceuticals
Cells
NOS
NO_3^-
NO_2^-, N_xO_y
Bioactivity
NO
NO_3^-
NO_2^-
Gut and Mouth Flora
Nitrate Reductase
Red Blood Cells
Hb, XOR, CA
NO_2^-
NO_3^-, NO_2^-, N_xO_y
HbFeNO, HbCysSNO
GSNO
Cells
CA
AMINES
N-Nitrosation
ONOH, N_2O_3
Nitrosoamines
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(A) NO-Bioactivity
Signalling (+)
Vasodilation (+)
Plateletaggregation (-)
GSNO
CysSNO
NO
(B) NO-Bioactivity
O- and N-Alkylation
Mutagenesis (+)
Cancerogenesis (+)
Kidney/Urine
Recycling of NO bioactivity
CA
Nitrite
CA
$U_{NOx}R$=[Nitrate]/[Nitrite]
Loss of NO bioactivity
Nitrate

Figure 1

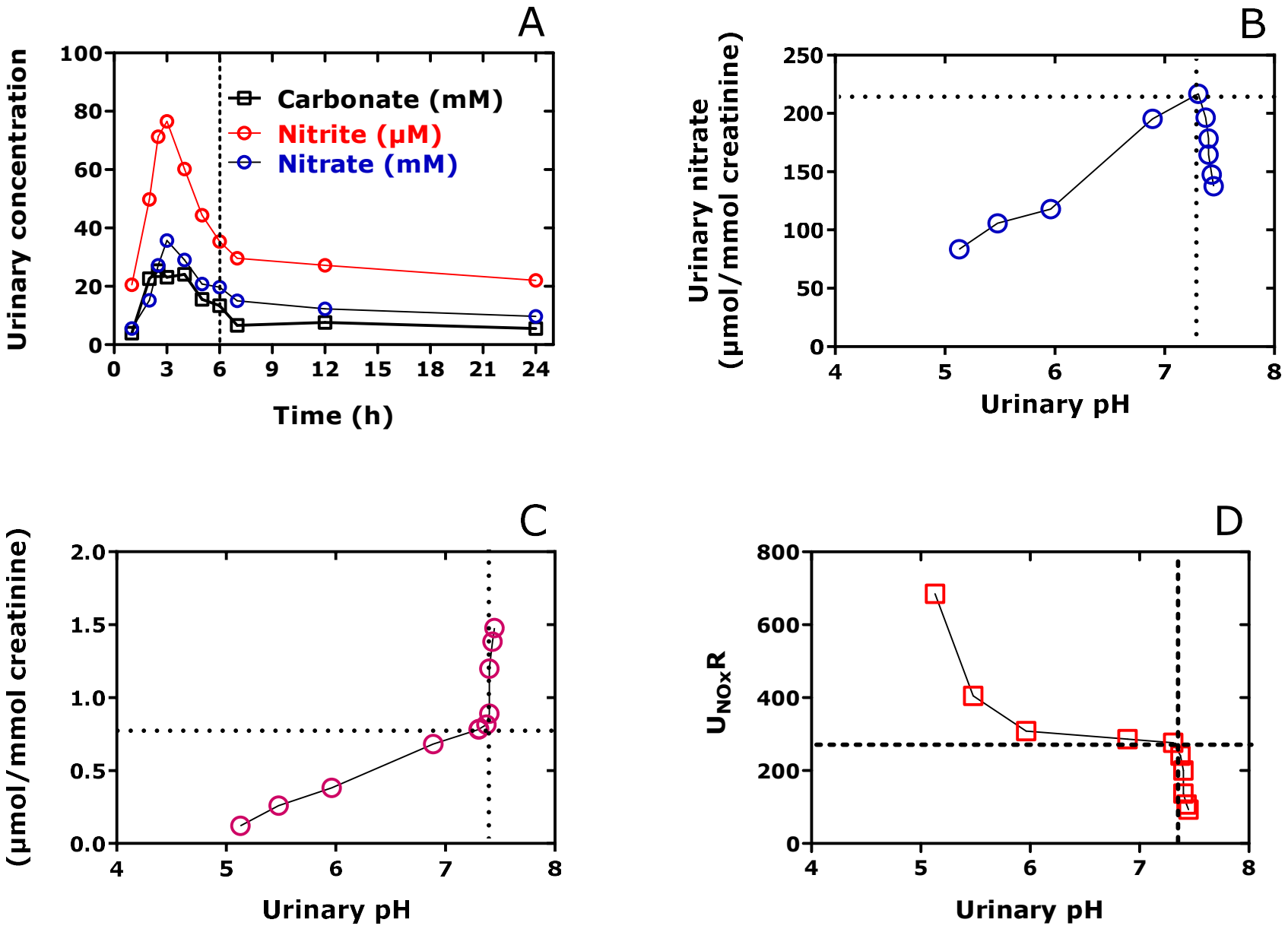
A
Urinary concentration
Carbonate (mM)
Nitrite (μM)
Nitrate (mM)
Time (h)
B
Urinary nitrate (μmol/mmol creatinine)
Urinary pH
C
Urinary nitrite (μmol/mmol creatinine)
Urinary pH
D
U_{NOxR}
Urinary pH

Figure 2

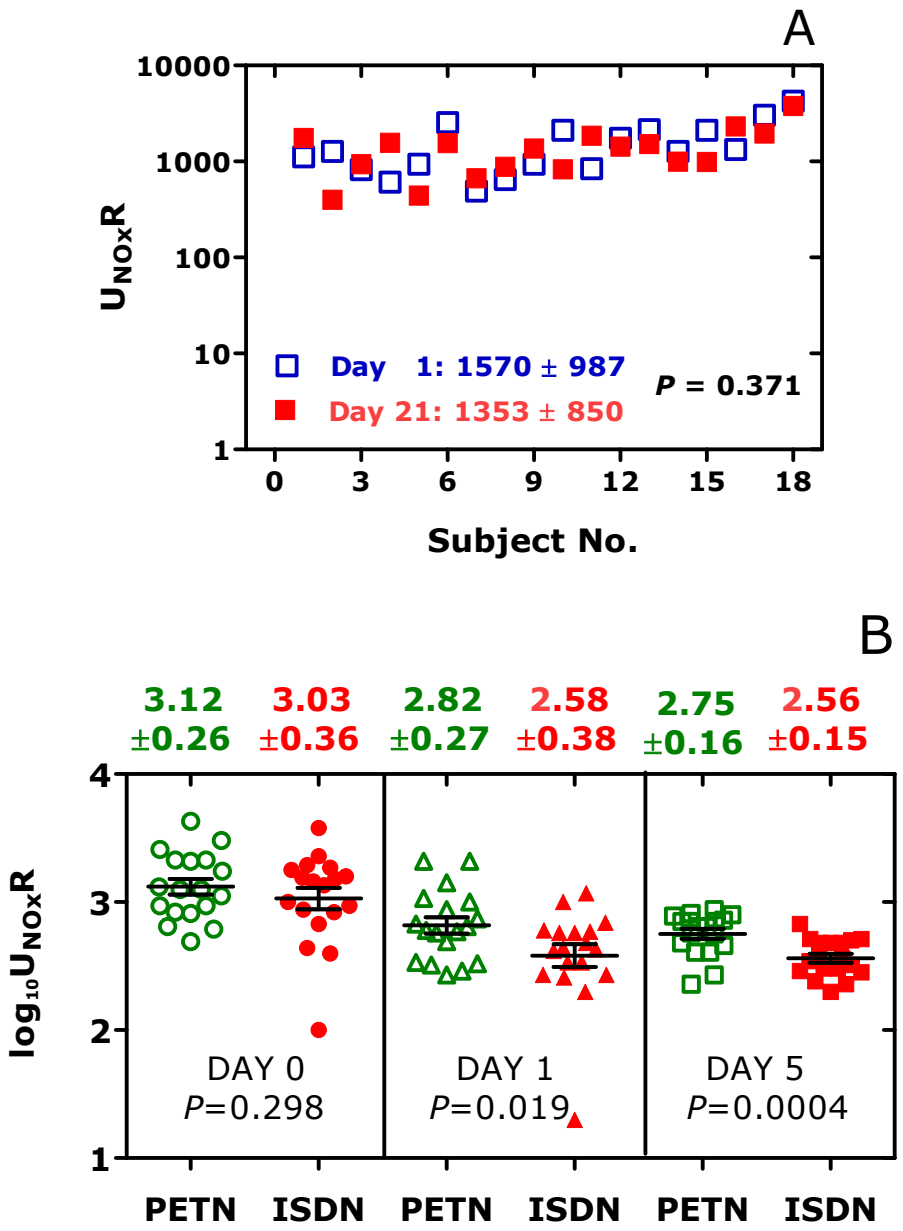
A
10000
1000
100
10
1
$U_{NOx}R$
Day 1: 1570 ± 987
Day 21: 1353 ± 850
P = 0.371
0
3
6
9
12
15
18
Subject No.
B
3.12 ±0.26
3.03 ±0.36
2.82 ±0.27
2.58 ±0.38
2.75 ±0.16
2.56 ±0.15
4
3
2
1
$log_{10}U_{NOx}R$
DAY 0
P=0.298
DAY 1
P=0.019
DAY 5
P=0.0004
PETN
ISDN
PETN
ISDN
PETN
ISDN

Figure 3

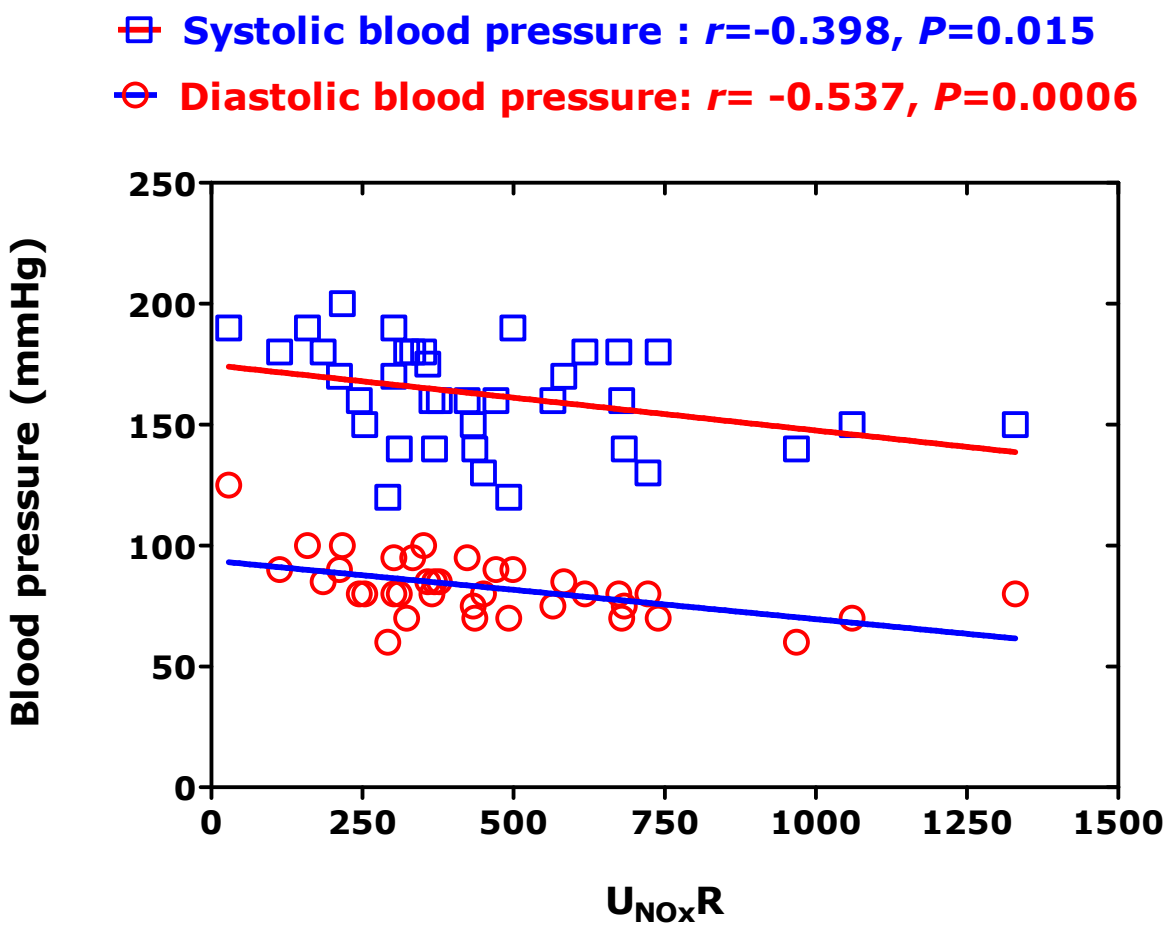
Systolic blood pressure : r=-0.398, P=0.015
Diastolic blood pressure: r= -0.537, P=0.0006
Blood pressure (mmHg)
250
200
150
100
50
0
0
250
500
750
1000
1250
1500
UNOxR

Figure 4

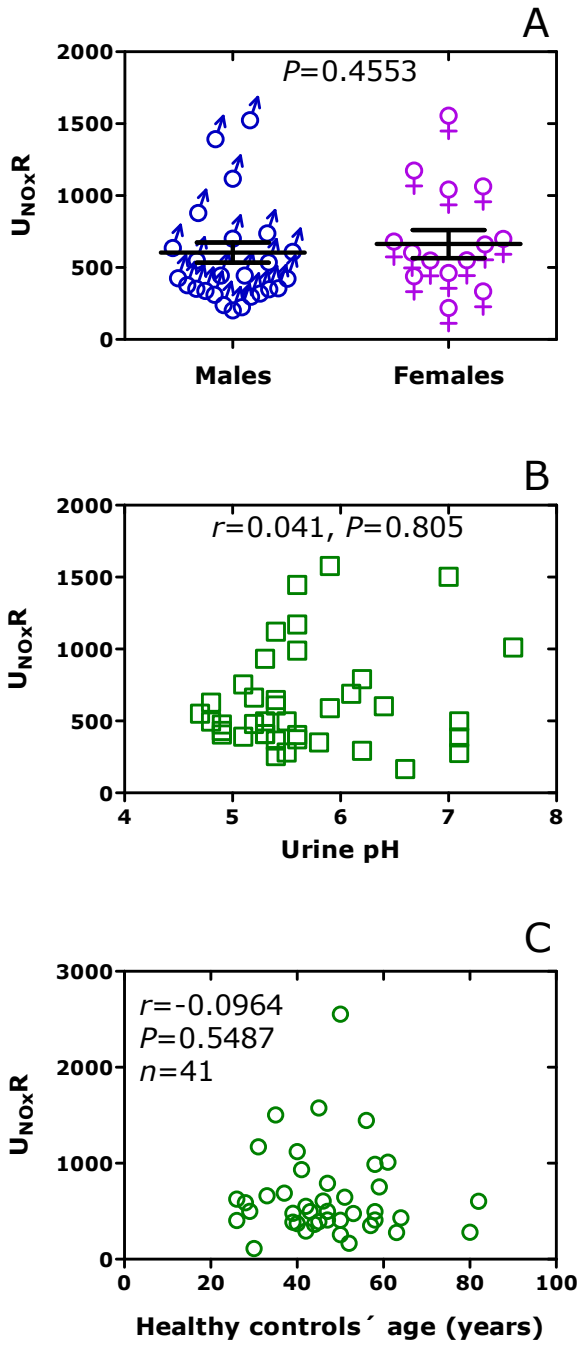
A
P=0.4553
2000
1500
1000
500
0
U_NOxR
Males
Females
B
r=0.041, P=0.805
2000
1500
1000
500
0
U_NOxR
4
5
6
7
8
Urine pH
C
r=-0.0964
P=0.5487
n=41
3000
2000
1000
0
U_NOxR
0
20
40
60
80
100
Healthy controls´ age (years)

Figure 5

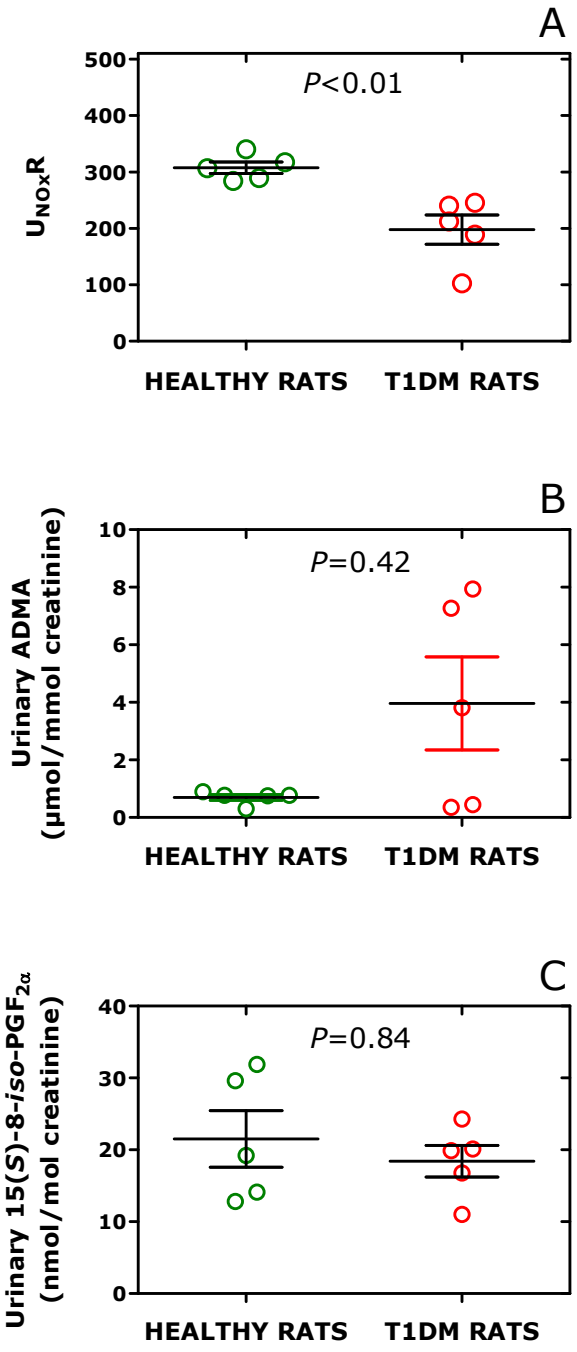
A
500
400
300
200
100
0
$U_{NOx}R$
P<0.01
HEALTHY RATS
T1DM RATS
B
10
8
6
4
2
0
Urinary ADMA
(µmol/mmol creatinine)
P=0.42
HEALTHY RATS
T1DM RATS
C
40
30
20
10
0
Urinary 15(S)-8-iso-$PGF_{2\alpha}$
(nmol/mol creatinine)
P=0.84
HEALTHY RATS
T1DM RATS

Figure 6

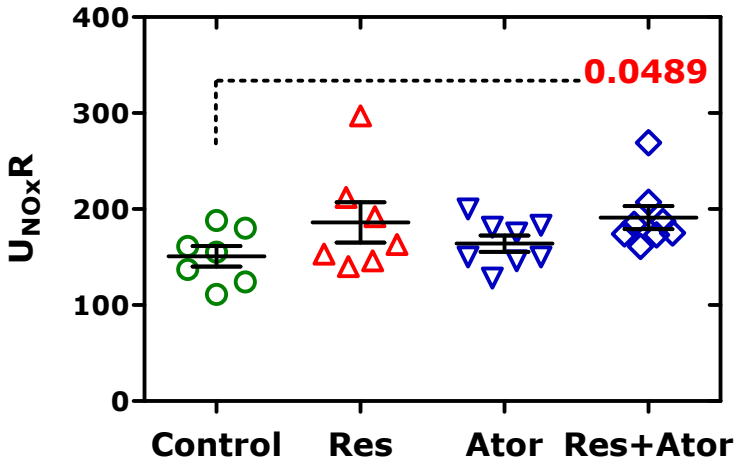

Figure 7A

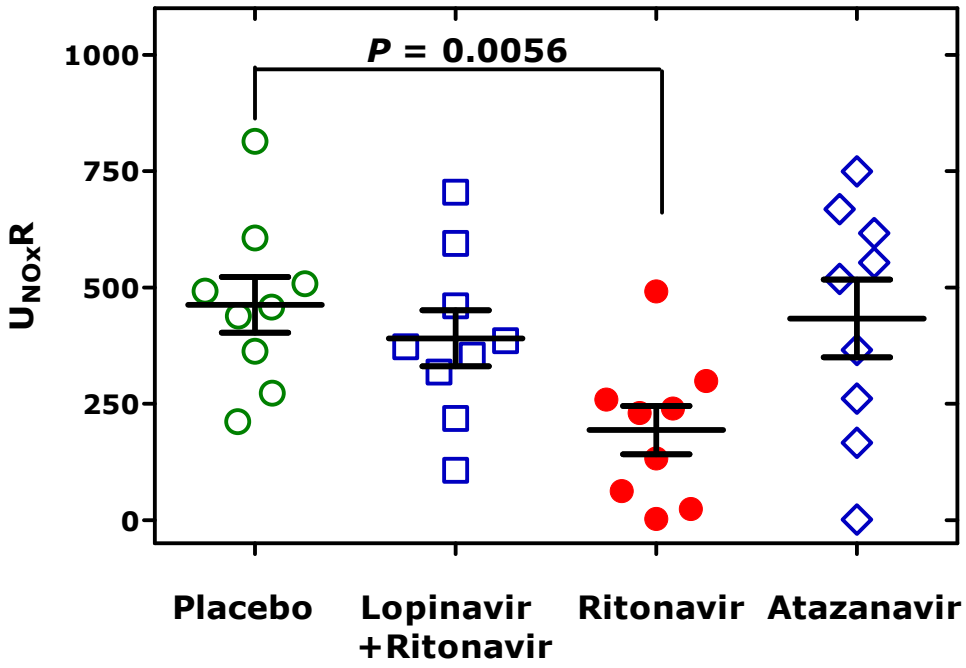
P = 0.0056
1000
750
500
250
0
U_NOxR
Placebo
Lopinavir +Ritonavir
Ritonavir
Atazanavir

Figure 7B

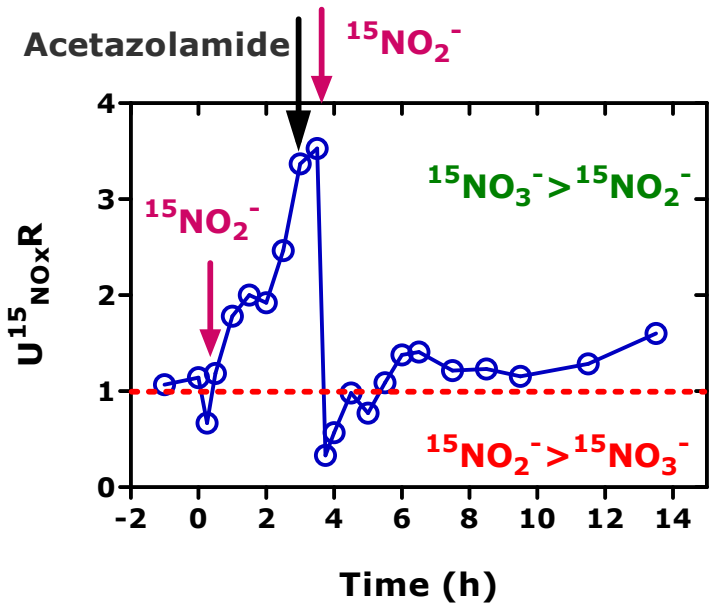
Acetazolamide
$^{15}NO_2^-$
$^{15}NO_2^-$
$^{15}NO_3^- > ^{15}NO_2^-$
$^{15}NO_2^- > ^{15}NO_3^-$
U^{15}_{NOxR}
Time (h)
0
1
2
3
4
-2
0
2
4
6
8
10
12
14

Figure 8A

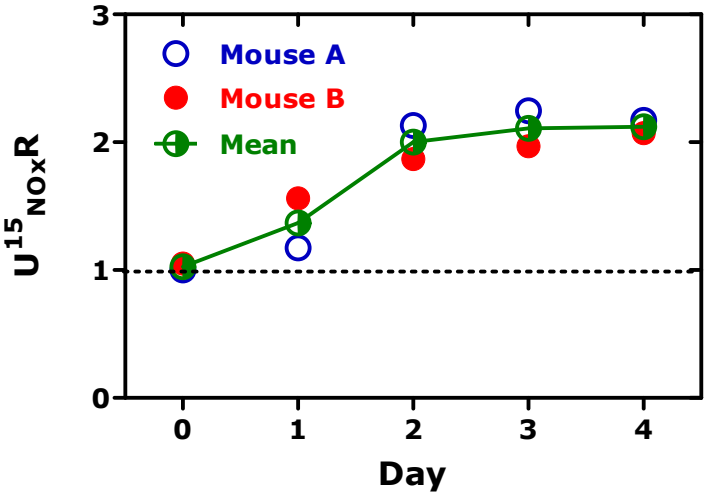

Figure 8B

Supplement to

Carbonic anhydrase-dependent reabsorption of nitrite in humans and animals: Results, meta-analysis and a first evaluation of $U_{NOx}R$, the urinary nitrate-to-nitrite molar ratio, in experimental and clinical settings

Dimitrios Tsikas[1,*], Erik Hanff[1], Alexander Bollenbach,[1] Ruan Kruger,[2] Vu Vi Pham[1], Kristine Chobanyan-Jürgens[3], Dirk Wedekind[4], Tanja Arndt[5], Anne Jörns[5], Jimmy F.P. Berbée[6,7], Hans M.G. Princen[8], Thomas Lücke[9], François Mariotti[10], Jean-François Huneau[10], Stefan Ückert,[11] Jürgen C. Frölich[1], Sigurd Lenzen[5]

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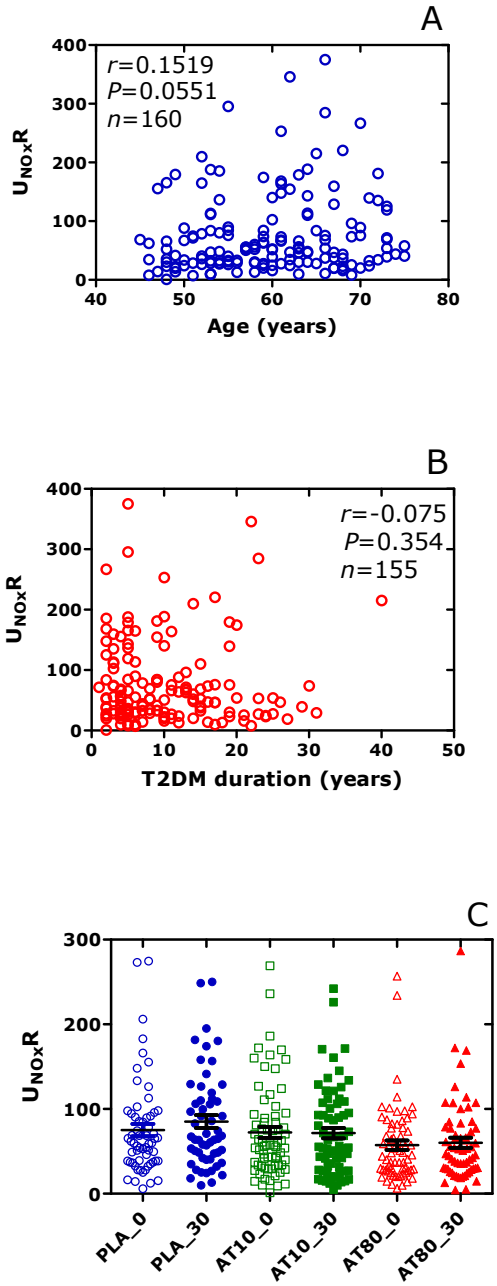

Fig. S1. Relationship between $U_{NOx}R$ and (A) age or disease duration (B) in the T2DM patients of the DALI study (The DALI Study Group 2001) at baseline. Effect of a 30-weeks (30) treatment with atorvastatin (AT) 10 mg/d (10), 80 mg/d (80) or placebo (PLA). This Figure was constructed with data reported in the Table 1 of a previous study (Tsikas et al. 2015).

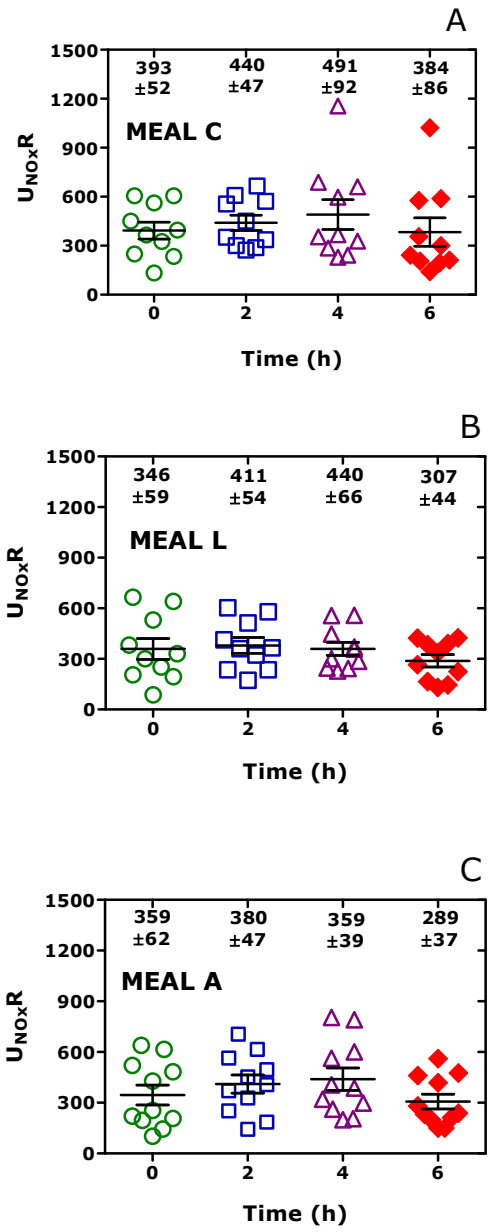

Fig. S2. $U_{NOx}R$ in 10 overweight men upon ingestions of three different mealsThe meals differed with respect to the protein isolates (casein in meal C, whey protein in meal A, and α-lactalbumin-enriched whey protein in meal L). Data are shown as mean±SEM. For more details see the main text.

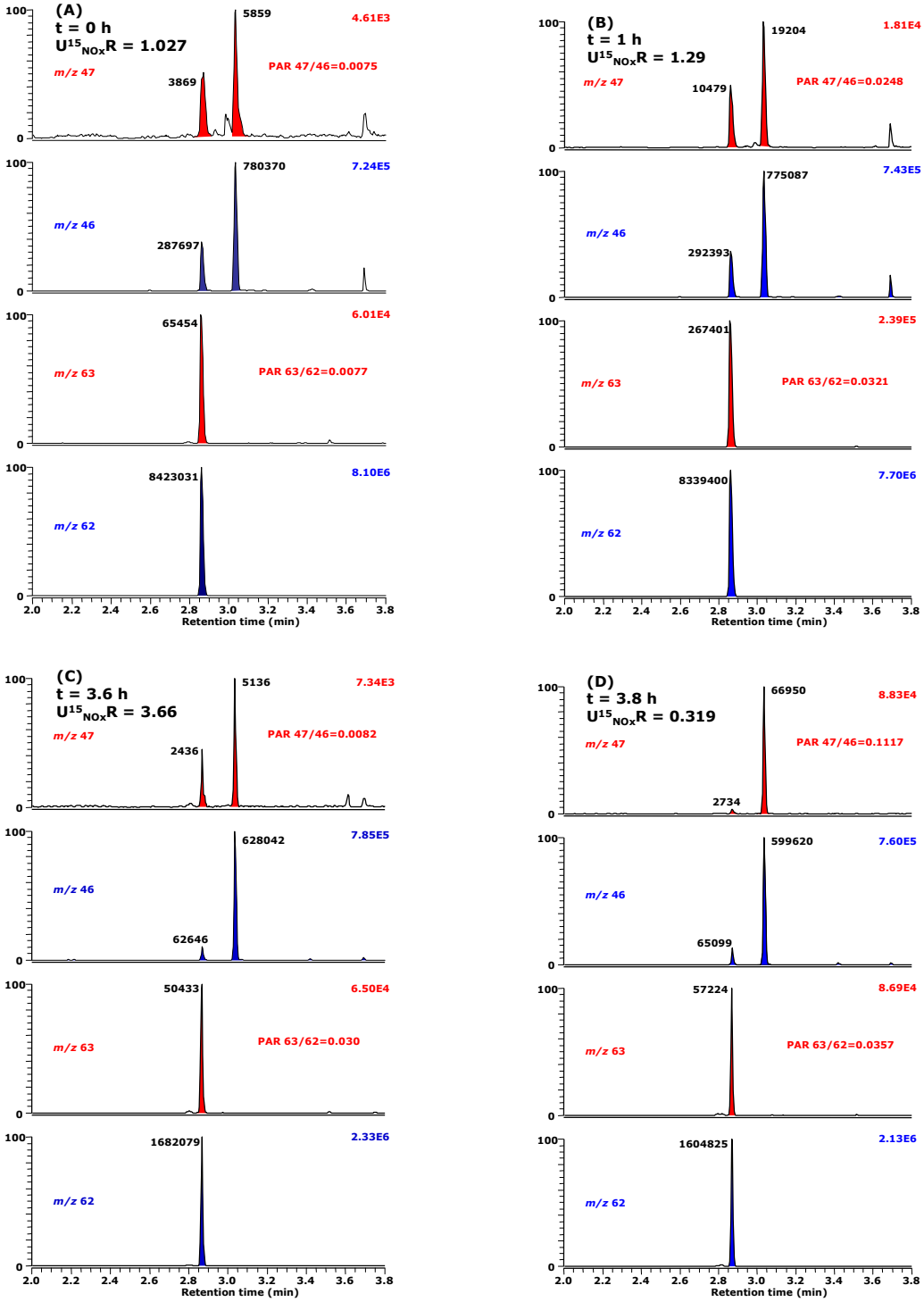

Fig. S3. GC-MS chromatograms from analyses of spot urine samples collected before and after [^{15}N]nitrite sodium and acetazolamide intake by a healthy female subject (age, 25 years) (Tsikas et al. 2010b). The volunteer drank two times [^{15}N]nitrite sodium dissolved in drinking water (0.31 μmol/kg each) and took once acetazolamide (5.4 mg/kg bodyweight). The first [^{15}N]nitrite intake was ingested at the time point "zero" (0 h, panel A). $U^{15}_{NOx}R$ is calculated by dividing the peak area ratio (PAR) of *m/z* 63

([^{15}N]nitrate) to *m/z* 62 ([^{14}N]nitrate) by the PAR of *m/z* 47 ([^{15}N]nitrite) to *m/z* 46 ([^{14}N]nitrite) (see Fig. 8A in the main text).

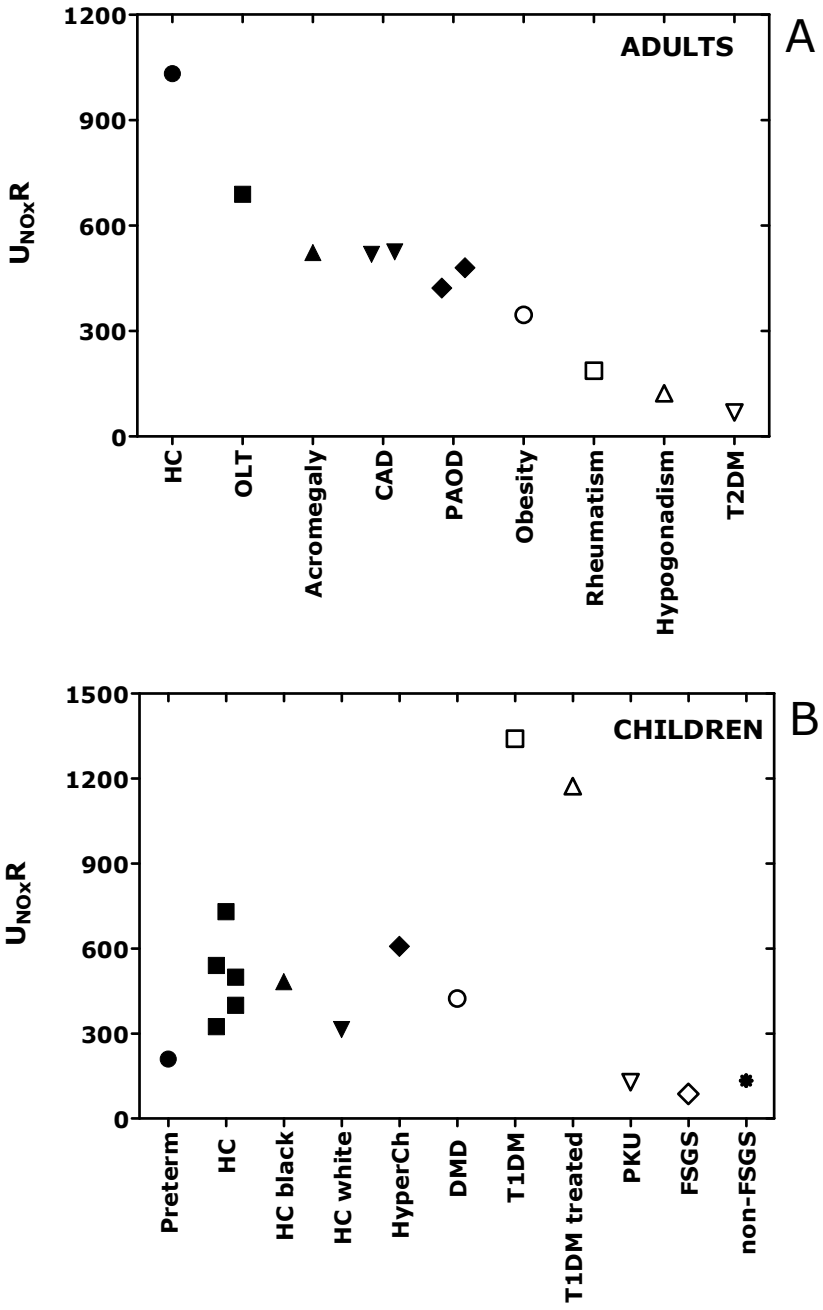

Fig. S4. Illustration of the $U_{NOx}R$ values for healthy and ill adults (A) and children (B) reported in the main text of the article (see Table 1). HC, healthy; OLT, patients with liver disease before orthotopic liver transplantation; HyperCh, hypercholesterolemia; DMD, Duchenne muscular dystrophy; PKU, phenylketonuria; FSGS, sporadic focal segmental glomerulosclerosis.