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ORIGINAL ARTICLE

Asymmetric dimethylarginine (ADMA) in human blood: effects of extended haemodialysis in the critically ill patient with acute kidney injury, protein binding to human serum albumin and proteolysis by thermolysin

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Abstract Free, non-protein bound asymmetrically guanidine-dimethylated arginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthesis. Human erythrocytic membrane comprises considerable amounts of large (>50 kDa) ADMA-containing proteins. Location in the erythrocyte membrane and identity and physiological functions of ADMA-containing proteins are unrevealed. In healthy subjects, the concentration of free ADMA in heparinised plasma is almost identical to that of serum. We hypothesised that the robustness of free ADMA concentration in human blood is due to a remarkable resistance of erythrocytic ADMA-containing proteins against proteases. In vivo, we investigated the course of the concentration of ADMA in serum and EDTA plasma of a critically ill patient with acute kidney injury during extended haemodialysis. In vitro, we studied the effects of thermolysin, a useful experimental proteolytic enzyme of erythrocyte membrane proteins, on erythrocytic ADMA. The protein binding (PB) of ADMA to human serum albumin (HSA) was also determined. In these studies, ADMA was measured by a previously reported, fully validated GC–MS/MS method. We measured almost identical ADMA concentrations in plasma and serum samples of the patient. During dialysis, the circulating ADMA concentration decreased slowly and moderately indicating removal of this substance, which was however much less than expected from its low molecular weight (202 Da) and high water solubility. After dialysis, circulating ADMA concentration increased again, a phenomenon called rebound, and ADMA reached higher levels compared to the baseline. The PB value of ADMA to HSA was about 30 %. This surprisingly high PB value of ADMA to HSA may be an explanation for the rather poor dialysance of ADMA. Washed human erythrocytes suspended in phosphate-buffered physiological saline were found not to release appreciable amounts of free and ADMA-containing proteins. The lack of effect of coagulation or anticoagulation on the concentration of circulating free ADMA in humans is likely to be due to a remarkable resistance of ADMA-containing proteins in the erythrocyte membrane against proteases in vivo in humans. Our study suggests that free ADMA is released in the circulating blood at relatively high rates. The considerable PB of ADMA to HSA is likely to add to the apparently poor dialysability of ADMA. Other contributing factors could be redistribution of free ADMA between plasma and erythrocytes in favour of plasma ADMA and parallel formation of free ADMA from erythrocytic ADMA-containing proteins during haemodialysis.

Keywords Anticoagulation · Coagulation · Erythrocytes · Haemodialysis · Mass spectrometry · Nitric oxide · Proteins

Abbreviations

ADMA	Asymmetric dimethylarginine (N^G,N^G-dimethyl-L-arginine)
CID	Collision-induced dissociation
DDAH	Dimethylarginine dimethylaminohydrolase
DMA	Dimethylamine
ECNICI	Electron-capture negative-ion chemical ionization

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GC–MS/MS	Gas chromatography-tandem mass spectrometry
HSA	Human serum albumin
NO	Nitric oxide
NOS	Nitric oxide synthase
PB	Protein binding
PBS	Phosphate buffer saline
SDMA	Symmetric dimethylarginine (N^G,N'^G-dimethyl-L-arginine)

Introduction

The asymmetrically guanidine (N^G)-dimethylated arginine (ADMA) occurs physiologically in human plasma as a free amino acid and in red blood cells both as a free amino acid and as a residue in proteins (Billecke et al. 2006; Zinellu et al. 2007, 2008; Billecke et al. 2009; D`Alecy and Billecke 2010; Davids et al. 2012; Großkopf et al. 2012). The free ADMA form is an inhibitor of the activity of constitutive and inducible nitric oxide synthase (NOS) isoforms (Tsikas et al. 2000; Kielstein et al. 2007). Free ADMA is generally regarded as the major endogenous regulator of NOS activity which converts L-arginine to nitric oxide (NO) and L-citrulline. Based on the concentration of ADMA in the circulation of adults, ADMA turned out to be a cardiovascular risk factor in various diseases (Lenzen et al. 2006). Chronic kidney disease in its most aggravated form, i.e. the need for chronic dialysis therapy, has been the first reported disease in which ADMA was found to accumulate in blood (Vallance et al. 1992). Although the origin of free ADMA in the circulation is not definitely resolved, ADMA-containing proteins are considered the exclusive source of free ADMA. Origin and physiological functions of ADMA-containing proteins in organs such as liver, kidney and brain are also incompletely understood.

Whole blood proteins contain approximately 4 μmol ADMA/g protein (Zinellu et al. 2008). Human serum albumin (HSA) and other plasma proteins (Tsikas and Beckmann 2009; Tsikas et al. 2010) are virtually free of covalently bound ADMA. Red blood cell proteins contain considerable amounts of ADMA covalently bound in their backbone (Zinellu et al. 2007, 2008; Billecke et al. 2006, 2009; D`Alecy and Billecke 2010). Human haemoglobin does not contain any covalently bound ADMA (Davids et al. 2012; Böhmer et al. 2012a). Thus, two of the most abundant proteins in blood cannot serve as sources of free ADMA, as it has been suggested previously (Kielstein et al. 2004). Location and identity of erythrocytic ADMA-containing proteins are still elusive. The biggest fraction of ADMA-containing erythrocytic proteins comprises proteins larger than 50 kDa (Großkopf et al. 2012).

A fraction of about 10 % of the daily produced free ADMA is excreted unchanged in the urine. The remaining ADMA fraction of around 90 % is eliminated in urine as dimethylamine (DMA) after hydrolysis of ADMA by dimethylarginine dimethylaminohydrolase (DDAH) (Leiper and Vallance 2006). Thus, DDAH in liver and kidney is primarily responsible for the elimination of ADMA. Originally, it has been reported that red blood cells possess both NOS (Kleinbongard et al. 2006) and DDAH activity (Kang et al. 2011). However, we and others found that human erythrocytes lack functional NOS (Böhmer et al. 2012b) and functional DDAH (Davids et al. 2012; Großkopf et al. 2012). These clinchers support the idea that ADMA may exert additional, still largely unrevealed, NO-unrelated physiological functions in erythrocytes and presumably in other cells. Characterisation and identification of ADMA-containing proteins in human erythrocytes may be a promising strategy to unveil ADMA's physiological roles in human circulation beyond inhibiting NOS activity.

Most information currently available for ADMA is based on the concentration of free ADMA measured by various different assays in plasma samples. The concentration range for ADMA in plasma of healthy adult humans is about 300 to 500 nM (Horowitz and Heresztyn 2007; Teerlink 2007). By means of a GC–MS/MS method, we determined a mean ADMA concentration of about 400 nM in plasma of healthy adults (Tsikas et al. 2003). Recently, it was reported that the concentration of free ADMA is practically the same in plasma and serum of healthy adults, while the concentration of L-arginine, the biochemical substrate of NOS, is different in plasma and serum samples of healthy adults (Davids et al. 2013). Different concentrations in human plasma and serum were also found for nitrite (Tsikas 2007), the autoxidation product of NO. Yet, effects of coagulation and anticoagulation of blood samples are not limited to the members of the L-arginine/NO pathway, but they are common to many other endogenous constituents. Coagulation and anticoagulation of blood may offer distinctly different values in plasma and serum for numerous biochemical parameters. The choice of plasma or serum may be of decisive importance in clinical-chemistry and clinical research studies. Although trivial, the use of the same coagulation/anticoagulation procedure and the same type of vacutainers including blood volume is imperative throughout a study to generate reliable and comparable results (Davids et al. 2013). In addition, harmonisation of blood sampling and coagulation/anticoagulation would allow a more reliable comparison of analytical data reported by different groups around the world. The consistency of free ADMA concentration in the circulation reported by evidently reliable analytical methods is most likely not unique but it is remarkable (Tsikas 2008).

The ADMA measurement in human blood is extraordinarily robust. A considerable amount of ADMA-containing proteins exists in erythrocytic membranes of human blood (Großkopf et al. 2012). Obviously, the distribution of free ADMA between plasma and erythrocyte cytosol and the contribution of the erythrocytic proteasome to free ADMA in lysed erythrocytes (D`Alecy and Billecke 2010; Davids et al. 2012) are well balanced. These observations prompted us to further investigate this phenomenon in vivo and in vitro in intact human erythrocytes.

ADMA has peculiar haemodialysis behaviour, and contradictory results have been reported for its dialysability (Schepers et al. 2014). We therefore quantitated ADMA by a previously reported, thoroughly validated GC–MS/MS method (Tsikas et al. 2003) in EDTA plasma and serum obtained in parallel from arterial blood of an adult critically ill patient suffering from acute kidney injury before, during and after extended haemodialysis. This secondary analysis was carried out on samples from previously reported studies (Burkhardt et al. 2009).

In vitro, we investigated the effect of the proteolytic enzyme thermolysin on the release of ADMA from human erythrocytes. Thermolysin (EC 3.4.24.27), a Zn^{2+}-containing Ca^{2+}-stabilized and thermo-stable peptidase, is widely distributed in nature (Adekoya and Sylte 2009) and is associated with hemorrhagic effects (Komori et al. 2001). Thermolysin possesses unique properties. For instance, it preferentially cleaves at the N-terminal side of hydrophobic or bulky amino side chains such as Leu, Phe, Ile and Val and can also hydrolyze dipeptides (Damoglou et al. 1971; Feder et al. 1971). In the past, thermolysin has been frequently used in biochemical studies on erythrocytes, for instance to characterise human erythrocyte proteins including the hexose transporter and the major protein (polypeptide 3) (Jenkins and Tanner 1977; Girg et al. 1981; Karim et al. 1987).

Eventually, we determined the protein binding of ADMA to HSA in aqueous buffer in order to test the possibility of whether plasma protein binding may explain the poor haemodialysability of ADMA (Kielstein et al. 2004).

Materials and methods

Materials and chemicals

The hydrochloride salt of asymmetric dimethylarginine (N^G,N^G-dimethyl-L-arginine, ADMA) and human serum albumin (fraction V, 96–99 % albumin; HSA) were purchased from Sigma (Deisenhofen, Germany). Thermolysin was obtained as a powder from Sigma-Aldrich (Munich, Germany). The supplier declared that the thermolysin preparation contained Ca^{2+}, but its content was not specified. Pentafluoropropionic anhydride was obtained from Pierce (Rockford, IL, USA). Tetradeuterated methanol (CD_3OD, 99.8 % at D) was supplied by Aldrich (Steinheim, Germany). All other chemicals including paracetamol (acetaminophen) and salicylic acid were obtained from Merck (Darmstadt, Germany). Amicon Ultra Millipore ultrafiltration cartridges (cut-off, 10 kDa) were supplied by Millipore Corporation (Bedford, MA, USA). Vivaspin cartridges (cut-off, 10 kDa) were obtained from Sartorius (Göttingen, Germany). A 30-m long fused-silica capillary column type Optima 17 (0.25 mm I.D., 0.25 µm film thickness) was purchased from Macherey–Nagel (Düren, Germany).

Human studies

Blood samples were drawn from the arterial line of the critically ill patient before, during and after an extended haemodialysis using a high-flux polysulfone F69 S dialyser (1.3 m^2) from Fresenius Medical Care (Germany). In addition, pre- and post-dialyser blood samples were taken. The Hannover Medical School Ethics Committee approved the study protocol (see Burkhardt et al. 2009). ADMA was measured in EDTA plasma and serum samples by GC–MS/MS (Tsikas et al. 2003). Data were examined by paired *t* test analysis, linear regression analysis, the Bland–Altman method (mean of the difference δ and the average μ) and the Oldham-Eksborg approach (the Oldham-Eksborg ratio ρOE) (Oldham 1968; Eksborg 1981; Bland and Altman 1986; Araujo 2009). Statistical analyses were performed using Microcal™Origin™ Version 5.0 (Microcal Software Inc, Northampton, USA).

Thermolysin study

Thermolysin solutions were freshly prepared in phosphate buffer saline (PBS) of pH 7.4. We abstained from the use of external Ca^{2+} for two reasons. First, because we knew that recombinant endothelial NOS, a strictly Ca^{2+}-dependent enzyme, externally added to washed erythrocytes suspended in PBS is active (Böhmer et al. 2012b). Second, it is also known that thermolysin-bound Ca^{2+} cannot be completely removed even with the use of gel filtration (Feder et al. 1971). We therefore assumed that the thermolysin used in our experiments was stable and functionally active.

A healthy volunteer donated blood which was collected by means of two heparin monovettes (Sarstedt, Germany). Blood samples were immediately centrifuged (800×*g*, 5 min, 4 °C), plasma and buffy coat were discarded, and erythrocytes were washed twice with physiological saline (4 mL). The two erythrocyte fractions were combined and then divided into two equal aliquots. One sample was incubated with 5 mL of PBS that contained thermolysin at a final concentration of 1 mg/mL. The second sample was incubated with 5 mL PBS that did not contain thermolysin

and served as a control. Both samples were incubated in parallel at 37 °C. Aliquots (each 1 mL) from artificial blood were taken immediately and subsequently at various incubation times and centrifuged immediately (800×*g*, 5 min, 4 °C). Free ADMA was determined in 10 µL aliquots of 10 kDa ultrafiltrate of the artificial plasma (upper PBS phase) without preceding HCl hydrolysis. For the determination of total ADMA (i.e. free ADMA plus ADMA covalently bound to proteins), aliquots (each 100 µL) of artificial plasma were added to 1000 µL aliquots of 6 M HCl from Merck (Darmstadt, Germany) in 1.3 mL glass vials which were locked gas-tightly and incubated for 24 h at 110 °C. The hydrolysate samples were ultrafiltered by centrifugation at room temperature (7500×*g*, 30 min) using Vivaspin cartridges. Two 10 µL aliquots of the ultrafiltrates were taken for derivatization and analysis using de novo synthesised trideuteromethyl-ADMA as the internal standard as reported elsewhere (Tsikas 2009). ADMA was quantitated by GC–MS/MS (Tsikas et al. 2003). For each incubation time point, the remaining erythrocytes and the remaining artificial plasma were gently mixed and added to the original incubation mixture.

This experiment was repeated with blood from the same donor, in the same way, yet with the following modifications. Blood was drawn using EDTA monovettes (Sarstedt, Germany). Thermolysin was used at 8 different concentrations (0–64 mg/mL); dilutions were performed with thermolysin-free PBS by starting from a 64-mg/mL thermolysin containing PBS sample. The incubation time was fixed to 2 h for all samples. Aliquots (0.5 mL) of artificial blood were processed.

Determination of the protein binding of ADMA to human serum albumin

The protein binding (PB) of ADMA to human serum albumin (HSA), i.e. the reaction (1a), was investigated by the ultrafiltration procedure as described previously for drugs (Kurz et al. 1977). For the sake of simplicity, reaction (1a) is re-written as reaction (1b) by substituting HSA by E and ADMA by A. In these reactions, n gives the number of ADMA molecules that bind to one HSA molecule.

$$\mathrm{HSA} + n\mathrm{ADMA} \leftrightarrow \mathrm{HSA} - \mathrm{ADMA}_n \tag{1a}$$

$$E + nA \leftrightarrow EA_n \tag{1b}$$

All experiments were performed in 67 mM potassium phosphate buffer, pH 7.4, at room temperature (about 23 °C) in duplicate, if not otherwise stated. To cover physiological and pathological concentrations in these experiments, the total ADMA concentration ($[\mathrm{ADMA}]_T$, C_{AT}) in the buffer (2 mL) varied between 0 and 2000 nM; the total concentration of HSA ($[\mathrm{HSA}]_T$, C_{ET}) in the experiments ranged between 0 and 60 g/L (i.e. 0 to about 880 µM). ADMA- and HSA-containing phosphate buffer solutions (4 mL) were incubated for 5 min at room temperature to allow equilibration. To measure the concentration of free ADMA at the equilibrium ($[\mathrm{ADMA}]_F$, C_{AF}) in these solutions, 2 mL aliquots were ultrafiltered by centrifugation for 1 min at 300×*g*. In some experiments, the remaining protein fractions of the samples (about 1.9 mL) were further ultrafiltered by centrifugation for 30 min at 7500×*g*. Two 10 µL aliquots of the ultrafiltrates were analysed for ADMA by GC–MS/MS (Tsikas et al. 2003). The ultrafiltrate volumes were less than 100 µL when centrifuged at 300×*g* and about 1.9 mL when centrifuged at 7500×*g*.

The equilibrium constant K_{EA} of the reaction (1a, 1b) and the PB of ADMA to HSA were calculated using the equilibrium concentrations of free HSA (C_{EF}), free ADMA (C_{AF}), bound ADMA (C_{AB}), total ADMA (C_{AT}) and the Formulae (2a–2e) and (3a–3c), respectively. Because bound ADMA is analytically not accessible, C_{AB} is calculated by difference: $C_{AB} = C_{AT} - C_{AF}$. C_{AF} is determined experimentally by measuring ADMA in the ultrafiltrate of gently (1 min, 300×*g*) centrifuged HSA-ADMA solutions in buffer. Because of the ultrafiltration technique, the ADMA concentration in the 300×*g* ultrafiltrate is considered to be the same as in the remaining protein fraction (Kurz et al. 1977).

$$K_{EA} = C_{EA_n} / \left[C_{EF} \times (C_{AF})^n \right] \tag{2a}$$

$$K_{EA} = C_{AB} / \left[C_{ET} \times (C_{AF})^n \right] \tag{2b}$$

$$K_{EA} = (C_{AT} - C_{AF}) / \left[C_{ET} \times (C_{AF})^n \right] \tag{2c}$$

$$C_{AT}/C_{AF} = 1 + K_{EA} \times C_{ET} \times (C_{AF})^{n-1} \tag{2d}$$

$$C_{AB} = \left(n \times C_{ET} \times (C_{AF})^n \right) / \left[1/K_{EA} + (C_{AF})^n \right] \tag{2e}$$

$$PB = \left[C_{EA_n} / C_{AT} \right] \times 100 \tag{3a}$$

$$PB = [C_{AB}/C_{AT}] \times 100 \tag{3b}$$

$$PB = [(C_{AT} - C_{AF})/C_{AT}] \times 100 \tag{3c}$$

Determination of the protein binding of paracetamol and salicylic acid to human serum albumin

These experiments were performed as described above for ADMA with the following modifications. The total concentration of HSA was 30 g/L. The PB values of paracetamol and salicylic acid to HSA were determined simultaneously in their mixtures with HSA at drug concentrations of 50, 100 and 250 µM each in 100 mM potassium phosphate buffer, pH 7.4, at 23 °C. Two 50 µL aliquots of the ultrafiltrates were diluted with 200 µL of the mobile phase

which was 45 mM $(NH_4)_2SO_4$-acetonitrile (90:10, v/v), and 200 µL aliquots thereof were analysed by reverse-phase HPLC with UV absorbance detection at 230 nm. Chromatographic separation was performed on a Gynkotek HPLC system (Germering, Germany) using a column (250 × mm i.d.) packed with a 5-µm Nucleodur C_{18} Gravity material from Macherey–Nagel (Düren, Germany) and a flow rate of 1 mL/min. The retention time was 6.2 min for paracetamol and 10.3 min for salicylic acid. Standard solutions of paracetamol and salicylic acid in the HSA-free buffer were used as calibrators.

GC–MS/MS conditions and analysis of ADMA

GC–MS/MS analyses in the electron-capture negative-ion chemical ionization (ECNICI) mode were performed on a triple-stage quadrupole (TSQ) mass spectrometer model ThermoQuest TSQ 7000 (Finnigan MAT, San Jose, CA) directly interfaced with a Trace 2000 series gas chromatograph equipped with an autosampler AS 2000 (CE Instruments, Austin, TX). The gas chromatograph was equipped with an Optima 17 fused-silica capillary column. An oven temperature programme was used with helium (at a constant flow rate of 1 mL/min) as the carrier gas: 1 min at 90 °C, then increased to 225 and 320 °C at a rate of 15 and 30 °C/min, respectively. Interface (280 °C), injector (280 °C) and ion-source (180 °C) were kept at constant temperatures. Electron energy was set to 200 eV and electron current to 300 µA. Methane (530 Pa) and argon (0.13 Pa collision pressure) were used as reagent and collision gases, respectively. Collision energy and electron multiplier voltage were set to 18 eV and 1.6 kV, respectively. Quantification was performed by selected-reaction monitoring of the mass transition *m/z* 634 → *m/z* 378 for unlabeled ADMA and *m/z* 637 → *m/z* 378 for the internal standard trideutero-ADMA with a dwell-time of 100 ms for each transition as described elsewhere (Tsikas et al. 2003).

Results and discussion

Circulating ADMA during extended dialysis of a critically ill patient

The results of the measurements of free ADMA in plasma and serum samples of the patient with acute kidney injury before, during and after dialysis and their statistical analysis are summarised in Table 1. For the sake of clarity, the

Table 1 Serum and plasma concentrations of ADMA measured by GC–MS/MS in one patient with acute renal failure before, during and after extended haemodialysis for 8 h

No.	Time (h)	Serum value V_{1j} (nM)	Plasma value V_{2j} (nM)	Bland–Altman average μ (nM)	Oldham/Eksborg difference δ (nM)	SR/PL ratio ρ_{OE}
1	0.0	1300	1290	1295	10	1.0077
2	0.5	1350	1390	1370	−40	0.9712
3	1.0	1410	1410	1410	0	1.0000
4	2.0	1440	1440	1440	0	1.0000
5	4.0	1510	1480	1495	30	1.0203
6	6.0	1490	1440	1465	50	1.0347
7	8.0	1560	1540	1550	20	1.0130
8 pd	12.0	410	400	405	10	1.0250
8	12.0	1560	1550	1555	10	1.0060
9	14.0	1420	1400	1410	20	1.0143
10	16.0	1340	1320	1330	20	1.0152
11	18.0	1300	1300	1300	0	1.0000
12 pd	20.0	300	300	300	0	1.0000
12	20.0	1410	1320	1365	90	1.0682
13	20.5	1480	1440	1460	40	1.0278
14	21.0	1530	1490	1510	40	1.0268
15	23.0	1690	1640	1665	50	1.0305
16	28.0	1820	1860	1840	−40	0.9785
Mean ± SD		1351 ± 386	1334 ± 383	1343 ± 384	17.2 ± 1.2	1.0133 ± 1.0219

Samples were analyzed in parallel. Data were analyzed by Bland–Altman, Oldham-Eksborg, linear regression (SR = 12.7 + 1.003 × PL, $r = 0.99673$) and t test ($P = 0.03168$) between SR and PL

SR serum, *PL* plasma, *pd* post-dialyzer

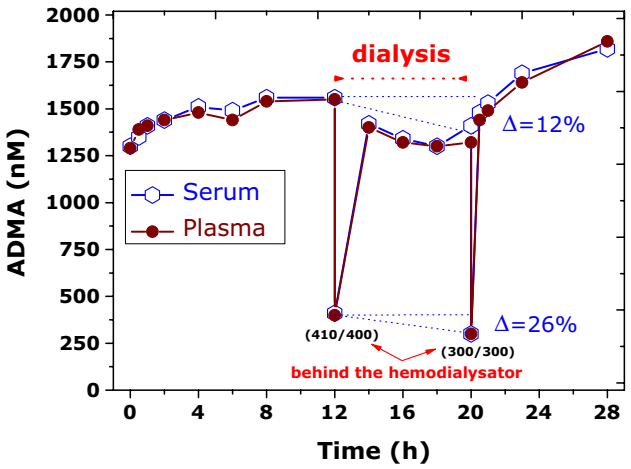

Fig. 1 Concentration of free ADMA in serum and plasma of a critically ill patient with acute renal failure undergoing extended haemodialysis for 8 h as indicated by the horizontal line. ADMA in plasma and serum was quantitated by GC–MS/MS as described previously (Tsikas et al. 2003)

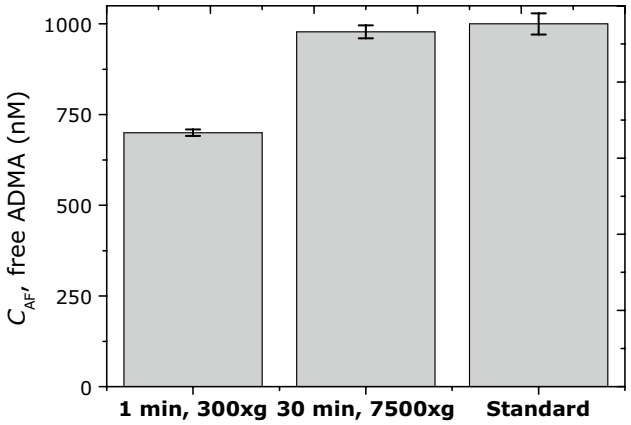

Fig. 2 Concentration of free ADMA in ultrafiltrates (cut-off, 10 kDa) obtained by centrifugation of a solution of ADMA (1000 nM) and HSA (30 g/L, about 440 μM) in 67 mM potassium phosphate buffer, pH 7.4, first at 300×*g* (sample "1 min, 300×*g*") and then at 7500×*g* (sample "30 min, 7500×*g*"), as well as by centrifugation of a HSA-free solution of ADMA (1000 nM) in 67 mM potassium phosphate buffer, pH 7.4 (sample "Standard"). ADMA was quantitated by GC–MS/MS as described previously (Tsikas et al. 2003). Data are presented as mean ± SD from 5 independent analyses. The ADMA concentrations measured in the ultrafiltrates were 700 ± 9, 978 ± 18 and 1000 ± 29 nM. From these data, a PB value of about 30 % of ADMA to HSA is estimated

course of the circulating ADMA concentration is displayed graphically in Fig. 1. The plasma and serum concentrations of free ADMA measured in the patient are throughout higher than the plasma and serum concentrations of free ADMA in healthy subjects (Horowitz and Heresztyn 2007; Teerlink 2007; Tsikas 2008; Davids et al. 2012, 2013; Tsikas et al. 2003). Comparison between serum ADMA and plasma ADMA concentration by all method-comparison approaches applied in this study indicates an overwhelming agreement between serum and plasma concentrations for free ADMA in the critically ill patient (Table 1). Paired *t* test analysis revealed statistically significant difference ($P = 0.03$) between serum and plasma ADMA concentration, yet the means deviate by only 1.3 %.

The circulating ADMA concentrations were only slightly decreased by haemodialysis for 8 h with an initial decrease rate of about 60 nM ADMA per hour. This slow decline occurred despite a marked difference comparing pre-dialyser and post-dialyser blood samples, the so-called arterio-venous difference (about 1000 nM) across the dialyser (Table 1). After the end of haemodialysis, the concentration of free ADMA in serum and plasma increased above the pre-dialysis values with an initial rate of about 86 nM ADMA per hour (Fig. 1). This peculiar dialysis behaviour of ADMA has been reported previously (Kielstein et al. 1999, 2004).

The findings in healthy humans (Davids et al. 2013) and the results of the present study in a critically ill patient suffering from acute kidney injury suggest that measurement of free ADMA will reveal the same values in serum, heparinized and EDTA plasma samples, and presumably in citrated plasma of healthy and severely diseased humans. Thus, blood coagulation/anticoagulation is not a relevant issue for the measurement of circulating free ADMA from the clinical-chemistry perspective. During blood sampling and generation of plasma or serum clinical-chemistry relevant haemolysis (Tsikas et al. 2012), ex vivo proteolysis (Davids et al. 2012) and elevated oxidative stress in erythrocytes (Böhmer et al. 2012a) may occur. The closeness of serum and plasma concentrations of free ADMA both in healthy subjects (Davids et al. 2013) and in our critically ill patient suggests that these factors do not alter appreciably the measurement of free ADMA in human blood. At least with respect to the L-arginine/NO pathway, where plasma and serum L-arginine (Davids et al. 2013) and nitrite (Tsikas 2007) concentrations differ considerably, the robustness of the measurement of free ADMA in human blood is remarkable.

Protein binding of ADMA, paracetamol and salicylic acid to HSA

The protein-binding experiments indicate that ADMA binds to HSA to a considerable degree. By means of the ultrafiltration technique, the PB value of ADMA (at 1000 nM) to HSA (30 g/L, about 440 μM) was calculated to be about 30 % (Fig. 2). From these data, it can be calculated that on average 1 mol HSA binds 0.68 mmol ADMA. Using the same ultrafiltration technique, we determined a similar PB value (27 %) for paracetamol (at 50 μM) to HSA (at

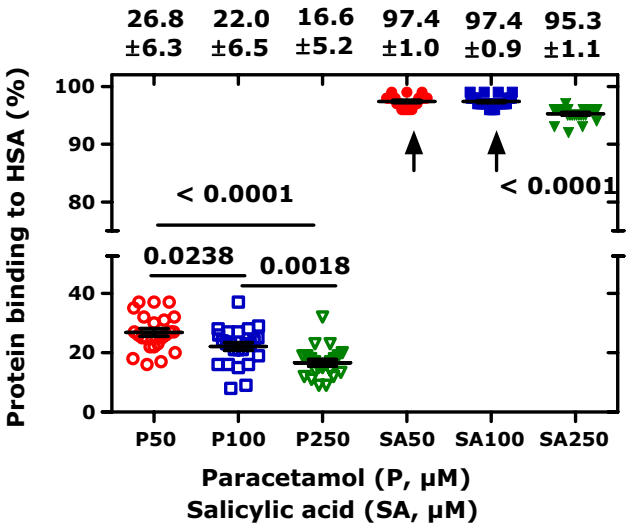

Fig. 3 Binding of paracetamol (P) and salicylic acid (SA) at 50, 100 and 250 µM each to HSA (at 30 g/L) in 100 mM potassium phosphate buffer, pH 7.4, at 23 °C

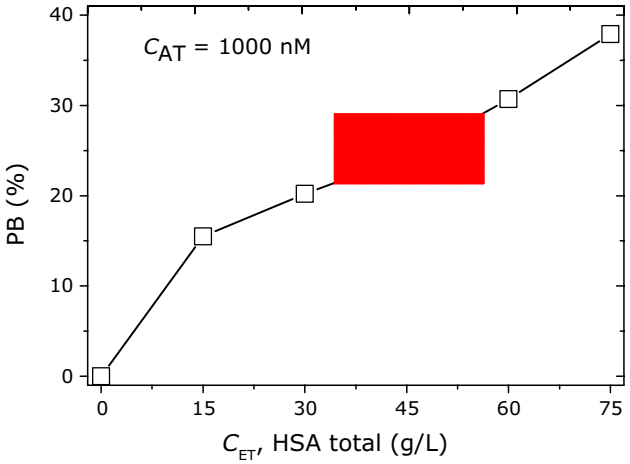

Fig. 4 Binding of ADMA (1000 nM) to HSA (range 0–75 g/L) in 67 mM potassium phosphate buffer, pH 7.4, at 37 °C. Marked area indicates the PB values for physiological plasma concentrations of HSA (i.e. about 35–55 g/L)

30 g/L) (Trettin et al. 2014), while the PB value of salicylic acid (at 50 µM) was much higher (97 %). At the fixed concentration of 30 g/L for HSA, the PB values of paracetamol and salicylic acid to HSA decreased with increasing concentration (Fig. 3). The PB values obtained in the present study agree very well with those reported in the literature for paracetamol of the order of 20 % (e.g. Morris and Levy 1984) and salicylic acid of the order of 96 % (e.g. Kramer and Routh 1973) to plasma or serum proteins including HSA. That very similar and slightly diverging ADMA concentrations were measured in the "30 min, 7500×*g*" and "1 min, 300×*g*" samples indicate the validity of the ultrafiltration approach used in the present study. Thus, the highly hydrophilic and at any physiological pH positively charged, asymmetrically N^G-dimethylated arginine analogue ADMA binds to HSA almost to the same degree as the quite lipophilic and electrically uncharged drug paracetamol (hydrophobicity cLogP, 1.29; Trettin et al. 2014), yet far less strongly than salicylic acid to HSA.

In the HSA concentration C_{ET} range of 0 to 75 g/L, the equilibrium concentration C_{AF} of ADMA decreased linearly upon increasing HSA concentration (data not shown). Plotting the ratio C_{AT}/C_{AF} against C_{ET} resulted in a straight line with an *y*-axis intercept of 1 and the slope value $K_{EA} \times (C_{AF})^{n-1}$ (2a). For a single-binding site of HSA for ADMA (i.e. $n = 1$), the K_{EA} of the reaction HSA + ADMA ↔ HSA-ADMA would be 486 M^{-1}. The PB value of ADMA (at 1000 nM) to HSA increased almost linearly in the range 15–75 g/L HSA (Fig. 4).

The PB of ADMA to HSA in 67 mM phosphate buffer, pH 7.4, was investigated for varying ADMA concentrations (C_{AT} = 0, 250, 500, 750, 1000 and 2000 nM) at a fixed HSA concentration of 60 g/L (C_{ET} = 60 g/L corresponding to about 880 µM). Linear relationships ($r > 0.999$) were obtained by plotting C_{AF} (in nM) against C_{AT} (in nM) for the "Standard" samples (i.e. $C_{ET} = 0$ g/L), for the samples "30 min, 7500×*g*" and for the samples "1 min, 300×*g*" with the regression equations $y = -28 + 1.20x$, $y = 24 + 1.09x$ and $y = -42 + 0.77x$, respectively. These findings confirm the PB value of about 30 % measured for C_{AT} = 2000 nM and C_{ET} = 30 g/L as reported above. Plotting C_{AB} (i.e. C_{AT}—C_{AF}, in nM) versus C_{AT} yielded a sigmoidal curve suggesting cooperative binding of ADMA to HSA (Fig. 5a). Plotting $1/C_{AB}$ (*y*) against $1/(C_{AF})^2$ (*x*) resulted in a straight line with the regression equation $y = 0.000265 + 1788x$ (Fig. 5b), suggesting that HSA has two binding sites for ADMA. From the slope value of the straight line [i.e. $1/K_{EA} \times n \times C_{ET} = 1788$ nM; see Formula (2e)] and for two identical binding sites of HSA for ADMA (i.e. $n = 2$), the equilibrium constant K_{EA} of the reaction HSA + 2 ADMA ↔ HSA-$(ADMA)_2$ is calculated to $K_{EA} = 3.2 \times 10^8$ M^{-2}. At a fixed concentration of HSA of 60 g/L, the PB value of ADMA to HSA increased with increasing ADMA concentration to reach maximum values in the range 20–30 % (Fig. 5c). In the physiological range for ADMA plasma concentration, the PB value ranged between 17 and 27 %.

The surprisingly high PB value of ADMA to HSA may be an explanation for the apparently poor haemodialysability of ADMA (Kielstein et al. 2004; Bouteldja et al. 2013). The PB of ADMA to HSA measured in the present study is supported by our findings in clinical microdialysis. In the microdialysate samples from skeletal and adipose tissues, the mean ADMA concentration was 270 nM and 170 nM ($P = 0.02$), respectively (May et al. 2014,

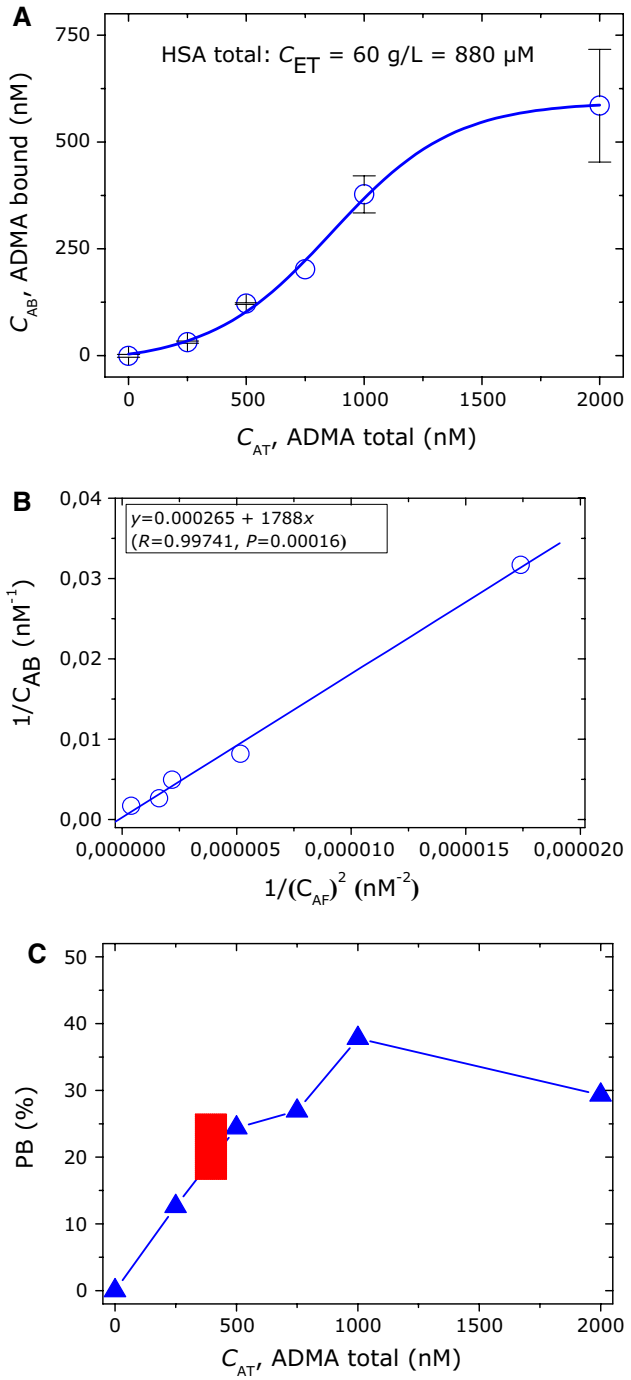

Fig. 5 Binding of ADMA (0–2000 nM) to HSA (60 g/L, about 880 µM) in 67 mM potassium phosphate buffer, pH 7.4, at 37 °C. **a** Relationship between the concentration of ADMA bound to HSA (i.e. C_{AB}) and the total concentration of ADMA (i.e. C_{AT}). **b** Relationship between the reciprocal concentration of ADMA bound to HSA (i.e. $1/C_{AB}$) and the reciprocal quadratic concentration of free ADMA (i.e. $(1/C_{AF})^2$). **c** PB value of ADMA to HSA in dependence on total ADMA concentration C_{AT}. Marked area in **c** indicates the PB values for physiological plasma concentrations of ADMA

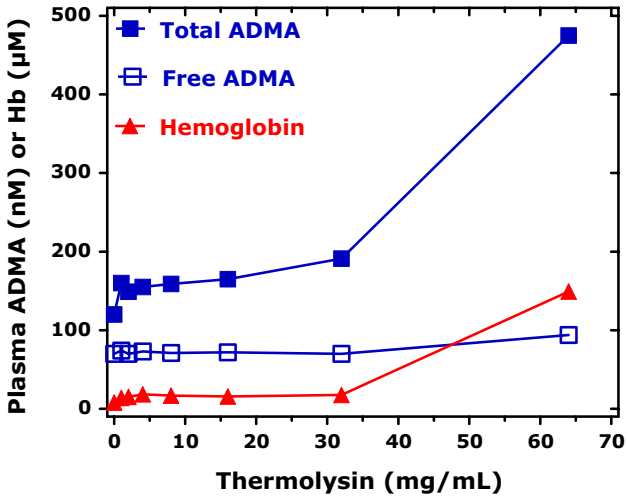

Fig. 6 Free (*open squares*) and total (*solid squares*) ADMA concentrations and haemoglobin (Hb) concentrations (*solid triangles*) in the artificial plasma (PBS phases) of washed human erythrocytes upon their incubation for 2 h at 37 °C with varying thermolysin concentrations. All analyses were performed on ultrafiltrates generated by centrifugation of artificial plasma (100 µL) using 10 kDa cut-off Vivaspin cartridges from Sartorius (Göttingen, Germany). Free ADMA was measured without HCl-catalysed hydrolysis. Total ADMA was measured in ultrafiltrates of HCl hydrolysates of supernatant fractions of both groups after incubation in 6 M HCl from Merck (Darmstadt, Germany) in 1.3 mL glass vials that were locked gas-tightly and incubated for 24 h at 110 °C. ADMA was quantitated by GC–MS/MS as described previously (Tsikas et al. 2003). Plasma Hb was measured spectrophotometrically (Tsikas et al. 2012)

2015). These ADMA concentrations are 2–3 times lower compared to those in plasma. However, when we consider that in the microdialysates the free concentration of ADMA was measured, whereas in the plasma the total ADMA concentration was measured, the total interstitial ADMA concentrations seem to be of the same order as in plasma. It is worth mentioning that HSA is also present in interstitial tissues, yet at concentrations lower than in serum: HSA concentration was determined to be 13.3 g/L in skeletal muscle, 7.4 g/L in adipose tissue and 48.9 g/L in of serum healthy humans (Ellmerer et al. 2000). Thus, HSA decreases the dialysability of ADMA in clinical microdialysis as well. The differences between skeletal and adipose tissues regarding ADMA concentration is microdialysates are likely to be due to different recovery rates which had not been determined in the studies (May et al. 2014, 2015).

An alternative explanation for the apparently low haemodialysance of ADMA could be the fact that human erythrocytes contain considerable amounts of free and protein-bound ADMA. Erythrocytes may release free ADMA to plasma in order to substitute the dialysed ADMA and to

re-establish the equilibrium between plasmatic and erythrocytic ADMA (Davids et al. 2012). Haemodialysis induces proteolysis (Ikizler 2005). Extended haemodialysis is likely to induce proteolytic activity in the red blood cells. Proteolysis of erythrocytic ADMA-containing proteins to free ADMA and its export into the plasma may be an additional mechanism simulating poor haemodialysance of ADMA.

Release of free ADMA by thermolysin-catalysed proteolysis of erythrocytic proteins

In lysed human erythrocytes, free ADMA concentration increases with incubation time; release of free ADMA is prevented by inhibiting proteolytic activity (Davids et al. 2012; Böhmer et al. 2012a). Studies from our group revealed that ADMA is covalently bound to ≥50-kDa large proteins of the erythrocyte membrane (Großkopf et al. 2012). In previous work, thermolysin turned out to be a useful proteolytic enzyme in studies on erythrocyte membrane proteins (Jenkins and Tanner 1977; Karim et al. 1987). In the present study, we used thermolysin in PBS to investigate whether proteolysis of proteins located on the outer surface of human intact erythrocytes may produce free ADMA and ADMA-containing peptides of a molecular mass larger than 10 kDa into the PBS. In the artificial plasma (i.e. PBS) of intact and washed erythrocytes in the absence or in the presence of thermolysin, the concentration of free ADMA increased almost in parallel over time (Fig. 6). These results indicate that the protease thermolysin at the concentration used had only little effect on the release of free and covalently protein-bound ADMA from intact erythrocytes. The small increases in free ADMA concentration seen in the artificial plasma may be due to transport of free ADMA from erythrocyte cytosol into the artificial plasma (Davids et al. 2012). Thermolysin, at the very high concentration of 64 g/L, increased considerably the concentration of ADMA-containing proteins in the artificial plasma, which was paralleled with an increase in haemoglobin concentration (Fig. 6). These findings may suggest that ADMA-containing proteins of the erythrocytic membrane are located on the outer surface of the erythrocyte membrane and are accessible to extracellular proteases, but they are very robust against proteolytic activity.

Conclusions

Erythrocytes of healthy humans contain free ADMA (about 400 nM) and ADMA covalently bound to erythrocytic proteins capable to release about 16 µM free ADMA upon complete proteolysis. The major fraction of ADMA-containing proteins in the erythrocyte membrane has a molecular mass larger than 50 kDa. The membrane of human erythrocytes is remarkably resistant against proteolytic enzymes such as thermolysin. Proteolysis and other phenomena that may occur during sampling, coagulation/anticoagulation as well as the centrifugation procedure (e.g. time, temperature, *g* number) of the blood do not contribute to variations of the concentration of free ADMA by more than about 1 % in healthy humans in vivo. Measurement of free ADMA in serum is as suited as in plasma from blood anticoagulated with clinical-chemistry relevant anticoagulants. Identity and physiological functions of ADMA-containing proteins in human blood remain to be elucidated.

Unexpectedly, ADMA has rather high PB values to HSA, which are in the same order of the PB value of the drug paracetamol (acetaminophen) to HSA. A PB value of ADMA to HSA of the order of 30 % is likely to contribute to the apparent poor haemodialysability of ADMA seen in the clinic, even using the so-called albumin dialysis technique (Rifai et al. 2010). The binding of ADMA to HSA seems to be complex and cooperative in nature. Delineation of the underlying binding mechanism may be helpful in improving the haemodialysability of ADMA. Export of free ADMA from erythrocytes into the plasma and erythrocytes and parallel formation of free ADMA from erythrocytic ADMA-containing proteins during haemodialysis are likely to add to the low haemodialysance of ADMA.

The focus of the present work was on ADMA. Symmetric dimethylarginine (SDMA) is another NOS activity inhibitor (Tsikas et al. 2000), which, like ADMA, circulates in blood both as free SDMA and as SDMA covalently bound to certain proteins. In contrast to ADMA, SDMA is exclusively eliminated by the kidney in its unchanged form. SDMA seems to be better dialyzable than ADMA (Bouteldja et al. 2013). Whether this is due to a lower plasma protein binding remains to be demonstrated. Given that both ADMA and SDMA accumulate in end-stage renal disease, with patients on dialysis presenting with the highest measured ADMA and SDMA levels (Schepers et al. 2014), efficient elimination of ADMA and SDMA in diseases such as end-stage renal disease is challenging and much-needed.

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

Ethical standard The studies involving human participants were approved by the Ethics Committee of the Hannover Medical School.

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