Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 858 (2007) 32–41
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

GC–MS assay for hepatic DDAH activity in diabetic and non-diabetic rats by measuring dimethylamine (DMA) formed from asymmetric dimethylarginine (ADMA): Evaluation of the importance of *S*-nitrosothiols as inhibitors of DDAH activity in vitro and in vivo in humans

Kristine Chobanyan [a], Thomas Thum [b], Maria-Theresia Suchy [a], Bijun Zhu [a], Anja Mitschke [a], Frank-Mathias Gutzki [a], Bibiana Beckmann [a], Dirk O. Stichtenoth [a], Dimitrios Tsikas [a,*]

[a] *Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany*
[b] *Department of Cardiology, Julius-Maximilians-University, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany*

Received 8 June 2007; accepted 2 August 2007
Available online 7 August 2007

Abstract

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthesis, is hydrolyzed to dimethylamine (DMA) and L-citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). In the present article we report on a GC–MS assay for DDAH activity in rat liver homogenate in phosphate buffered saline. The method is based on the quantitative determination of ADMA-derived DMA by GC–MS as the pentafluorobenzamide derivative. Quantification was performed by selected-ion monitoring of the protonated molecules at *m/z* 240 for DMA and *m/z* 246 for the internal standard $(CD_3)_2NH$ in the positive-ion chemical ionization mode. The assay was applied to determine the enzyme kinetics in rat liver, the hepatic DDAH activity in streptozotocin-induced (50 mg/kg) diabetes in rats, and to evaluate the importance of *S*-nitrosothiols as DDAH inhibitors. The K_M and V_{max} values were determined to be 60 μM ADMA and 12.5 pmol DMA/min mg liver corresponding to 166 pmol DMA/min mg protein. Typical DDAH activity values measured in rat liver homogenate were 8.7 pmol DMA/min mg liver at added ADMA concentration of 100 μM. DDAH activity was found to be 1.7-fold elevated in diabetic as compared to non-diabetic rats ($P = 0.01$). The SH-specific agents $HgCl_2$, *S*-nitrosocysteine ethyl ester (SNACET), a synthetic lipophilic *S*-nitrosothiol, *S*-nitrosoglutathione (GSNO), *S*-nitrosocysteine (CysNO) and *S*-nitrosohomocysteine (HcysNO) were found to inhibit DDAH activity in rat liver homogenate. The IC_{50} values for HcysNO, SNACET, CysNO and GSNO were estimated to be 300, 500, 700 and 1000 μM, respectively. Oral administration of ^{15}N-labelled SNACET to two healthy volunteers (1 μmol/kg) resulted in elevated urinary excretion of ^{15}N-labelled nitrite and nitrate, but did not reduce creatinine-corrected excretion of DMA in the urine. Our results suggest that inhibition of DDAH activity on the basis of reversible nitros(yl)ation or irreversible *N*-thiosulfoximidation of the sulfhydryl group of the cysteine moiety involved in the catalytic process is most likely not a rationale design of DDAH inhibitors. A major advantage of the present GC–MS assay over other assays is that DDAH activity is assessed by measuring the formation of the specific enzymatic product DMA but not the formation of unlabelled or (radio)labelled L-citrulline or the decay of the substrate ADMA. The GC–MS assay reported here should be suitable to probe for DDAH activity in various disease models.

Keywords: Diabetes; Enzyme inhibition; Michaelis–Menten; Nitric oxide; *S*-Nitrosothiols; SNACET

1. Introduction

Dimethylamine [DMA, $(CH_3)_2NH$)] is present in human blood and urine [1–3]. Special attention has been paid to DMA since its identification as the hydrolysis product of N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) [4]. ADMA is an endogenous inhibitor of nitric

* Corresponding author. Tel.: +49 511 532 3959; fax: +49 511 532 2750.
E-mail address: tsikas.dimitros@mh-hannover.de (D. Tsikas).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.08.002

oxide (NO) synthesis from L-arginine [5]. The enzyme N^G,N^G-dimethyl-L-arginine dimethylaminohydrolase (DDAH; EC 3.5.3.18) occurs in tissue, including liver and kidney, and hydrolyzes ADMA to yield DMA and L-citrulline [4] (Fig. 1A). ADMA has been shown to be actively metabolized to DMA by DDAH in humans in vivo [6].

In previous work we have reported on GC–MS methods for the quantitative determination of DMA in human plasma and serum [2] and in urine [3]. These methods are based on the simultaneous derivatization of DMA by pentafluorobenzoyl chloride to its pentafluorobenzamide derivative and extraction into toluene (Fig. 1B). We tested the applicability of these methods to assess DDAH activity by measuring the specific reaction product DMA in tissue. The method previously developed for circulating rather than for urinary DMA was found to be more appropriate for use in tissue homogenate. In the present work, we report on the development of a GC–MS-based DDAH assay for rat liver. Other assays assess DDAH activity by measuring the decay of unlabelled and labelled ADMA concentration [7,8], or the formation of ^{14}C-labelled L-citrulline from ^{14}C-labelled ADMA [9] or from ^{14}C-labelled L-monomethyl-arginine (^{14}C-L-NMMA) serving as the substrates for DDAH [10,11]. We applied this GC–MS assay to study the kinetics of the DDAH-catalyzed hydrolysis of ADMA to DMA in rat liver tissue. As DDAH activity is thought to be affected by oxidative stress which is generally believed to be elevated in diabetes, we determined, in the present study, hepatic DDAH activity in diabetic and non-diabetic rats. The effect of two classes of sulfhydryl-specific agents, i.e. $HgCl_2$ [9] and different *S*-nitrosothiols including *S*-nitroso-*N*-acetylcysteine ethyl ester (SNACET), a synthetic lipophilic *S*-nitrosothiol [12], on DDAH activity was also investigated in vitro and in vivo (i.e. using SNACET) in humans.

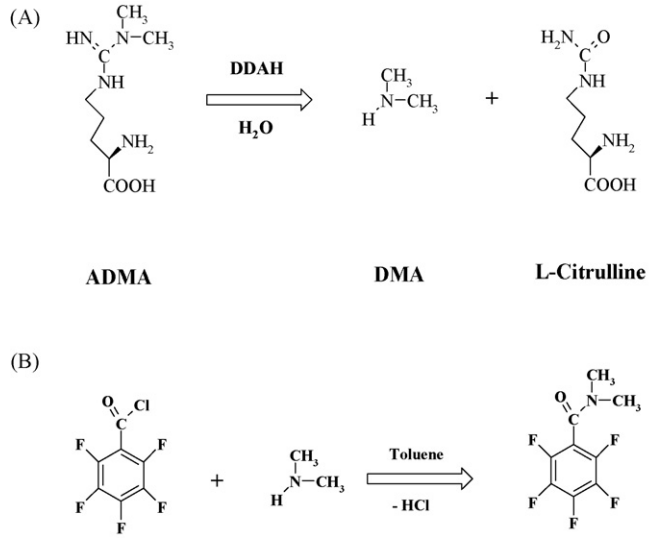

Fig. 1. (A) Conversion of asymmetric dimethylarginine (ADMA) to dimethylamine (DMA) and L-citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH; EC 3.5.3.18). (B) Reaction of pentafluorobenzoyl chloride with DMA to form the pentafluorobenzamide derivative.

2. Experimental

2.1. Materials and chemicals

2,3,4,5,6-Pentafluorobenzoyl chloride, the hydrochloride salts of unlabelled dimethylamine (d_0-DMA), hexadeutero-dimethylamine (d_6-DMA; declared as 99 at% ^{2}H), the sodium salt of ^{15}N-labelled nitrite (98 at% at ^{15}N), glutathione, cysteine, homocysteine, and a protease inhibitor mixture (PIM) were obtained from Aldrich (Steinheim, Germany). PIM was supplied as a solution in DMSO and consisted of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, *N*-(*trans*-epoxysuccinyl)-L-leucin-4-guanidinobutylamide, leupeptide hemisulfate, and pepstatin A. PIM was used as obtained from the manufacturer. Toluene and acetonitrile were purchased from Baker (Deventer, The Netherlands). Sodium carbonate and $HgCl_2$ were obtained from Merck (Darmstadt, Germany). Synthesis, purification and structural characterization of *N*-acetylcysteine ethyl ester (NACET) were described previously [12]. Unlabelled SNACET and *S*-[^{15}N]nitroso-SNACET (S^{15}NACET) were synthesized by mixing equal volumes of freshly prepared equimolar, aqueous, ice-cold solutions of NACET and nitrite ([^{15}N]nitrite) followed by acidification with 5 M HCl (final HCl concentration, 25 mM) [12]. Same procedures were used to synthesize *S*-nitrosoglutathione (GSNO), *S*-nitrosocysteine (CysNO) and *S*-nitrosohomocysteine (HcysNO). Stock solutions and dilutions of all *S*-nitrosothiols were stored in an ice-bath until use.

2.2. DDAH assay for rat liver homogenate

DMA is volatile and potentially unstable in biological samples, and therefore special precautions for sample collection and storage were taken. Stock solutions (each 100 mM) of d_0-DMA and d_6-DMA were prepared in 50 mM HCl and stored in stoppered flasks in a refrigerator (8 °C). Dilutions were prepared in distilled water and stored in ice during sample treatment. Analyses in this study were performed at least in duplicate; the number of replicates is reported in the respective experiments. Data are presented as mean ± SD.

Phosphate buffered saline (PBS, 100 mM, pH 7.1) was used for manually performed liver homogenization and DDAH assays. Liver homogenate was freshly prepared in ice-cold PBS using frozen 100- to 500-mg liver pieces to obtain liver tissue content up to 100 mg/ml. Remaining homogenate was discarded. Homogenates were used immediately after preparation and stored in an ice-bath until use. Protein concentration in clear supernatants of diluted homogenates (1:200, v/v) was determined by the method of Bradford [13]. With exception of the study on DDAH activity in diabetic and non-diabetic rats, protein concentration was determined representatively in three freshly prepared homogenates because a single liver was used in the whole study. In three rat liver homogenates with a tissue content of approximately 100 mg/ml, a protein concentration of 7.5 ± 0.1 mg/ml was measured. All incubations (usually sample volume of about 130 μl) were performed in 1.5-ml polypropylene tubes at 37 °C. The commercially available PIM solution in

DMSO was used without dilution. Following the suggestion of a previous study [8], we used in the present work PIM in order to inhibit formation of ADMA from hydrolysis of liver proteins during incubation. The volume ratio of sample to PIM solution was about 10:1. Neither DMSO nor PIM (each added up to 10 vol.%) were found to interfere with the quantitative GC–MS analysis of DMA in this assay (data not shown). Enzymatic reaction was stopped by immediate sample derivatization/extraction after the respective incubation time (see Section 3). More experimental details of the DDAH assay are reported in the subsequent subsections.

2.2.1. Sample derivatization and extraction

Because tissue homogenate in PBS resembles plasma or serum rather than urine with respect to protein concentration, DMA concentration expected to be formed, and pH, the derivatization/extraction procedure used in the present study is almost identical with that used for plasma and serum samples previously [2]. Samples were vortex-mixed manually using a Heidolph vortex mixer model Reax 2000 (Schwabach, Germany) at the highest vortex speed (i.e. stage 9). Immediately after completed incubation, toluene (1000 μl), Na_2CO_3 (10 μl of 20 mM) and pentafluorobenzoyl chloride (10 μl of a 10 vol.% solution in acetonitrile) were added in this order, and samples were mixed for 5 min by vortexing. After centrifugation (5 min, 800 × *g*, 4 °C) an aliquot (700 μl) of the organic phase was transferred into a 1.5-ml autosampler glass vial for GC–MS analysis. In case of clouding decanted toluene samples should be centrifuged again. Routinely, the samples were analyzed on the same day overnight.

2.2.2. Intra-assay accuracy and precision for DMA in rat liver homogenate

To a 4800-μl aliquot of ice-cold PBS, a 600-μl aliquot of PIM and a 600-μl aliquot of a 100-μM solution of ice-cold d_6-DMA were added and vortexed. The resulted mixture was used to homogenize a 450-mg piece of ice-cold rat liver under cooling. There were taken 100-μl aliquots of the homogenate and put in an ice-bath. Subsequently, samples were spiked with 10-μl aliquots of DMA solutions in distilled water (0–260 μM) to achieve final added DMA concentrations of 0–26 μM and put in an ice-bath for 10 min. Eventually, samples were derivatized/extracted as described above. All analyses were performed in quadruplicate.

2.2.3. Time-dependence of DMA formation in liver homogenate

A 500-mg piece of ice-cold rat liver was homogenized in a 6-ml aliquot of ice-cold PBS. To 100-μl aliquots of the homogenate were added 10-μl aliquots of a 100-μM solution of ice-cold d_6-DMA, 10-μl aliquots of ADMA solutions in distilled water to achieve added final concentrations of 0, 25, 50, 75 and 100 μM, and 10-μl aliquots of PIM. The samples were put in an ice-bath for 10 min. After this time two samples were derivatized/extracted immediately as described above. The other samples were incubated at 37 °C for 15, 30, 60 and 90 min. After the respective incubation time, samples were derivatized/extracted as described above. All samples were analyzed in duplicate.

2.2.4. Dependence of DDAH activity upon protein concentration

To a 3360-μl aliquot of ice-cold PBS, a 420-μl aliquot of PIM and a 420-μl aliquot of a 100-μM solution of ice-cold d_6-DMA were added and vortexed. By means of a 1.4-ml aliquot of the resulting mixture a 140-mg piece of ice-cold rat liver was homogenized under cooling. This homogenate was diluted with a mixture of PIM (final fraction 10 vol.%) and d_6-DMA (final concentration 10 μM) in ice-cold PBS to obtain homogenates with final tissue contents of 75, 50, 25, 10, and 0 mg/ml. There were taken 100-μl aliquots of the homogenates and put in an ice-bath for 10 min. Then samples were incubated for 5 min at 37 °C. Reaction was started by addition of 10-μl aliquots of an ADMA solution in distilled water (400 μM) to achieve a final added concentration of 40 μM. After 15 min of incubation, samples were derivatized/extracted as described above. All samples were analyzed in quadruplicate.

2.2.5. Determination of the Michaelis constant

To a 3200-μl aliquot of ice-cold PBS, a 400-μl aliquot of PIM and a 400-μl aliquot of a 100-μM solution of ice-cold d_6-DMA were added and vortexed. By means of this solution a 300-mg piece of ice-cold rat liver was homogenized under cooling. There were taken 90-μl aliquots of this homogenate and put in an ice-bath for 10 min. Each of the four samples was incubated for 5 min at 37 °C. Reaction was started by addition of 10-μl aliquots of ADMA solutions in distilled water (0–6000 μM) to achieve final added concentrations in the range of 0–600 μM. After 15 min of incubation, samples were derivatized/extracted as described above. The Michaelis constant K_M and the maximum specific activity (V_{max}) of DDAH were determined using the Lineweaver–Burk plot.

2.2.6. Effect of sulfhydryl-specific agents on DDAH activity

A 460-mg piece of ice-cold rat liver was homogenized in a 4.14-ml aliquot of ice-cold PBS containing 10 vol.% PIM and 10 μM d_6-DMA. There were taken three 1200-μl aliquots and put in an ice-bath. To the sample serving as the control a 200-μl aliquot of PBS was added. To the second sample a 180-μl aliquot of PBS and a 20-μl aliquot of an aqueous 10 mM $HgCl_2$ solution was added to reach a final $HgCl_2$ concentration of 143 μM. Eventually, the third sample was spiked with a 200-μl aliquot of a freshly prepared aqueous solution of SNACET (2 mM) to reach a final concentration of 286 μM. After an initial incubation of all samples for 3 min at 37 °C, reaction was started by adding to each sample a 140-μl aliquot of an aqueous solution of ADMA (1000 μM), finally yielding an initial ADMA concentration of 91 μM each. Samples were incubated for 0, 15, 30, 60 and 90 min. After the respective incubation time, samples were derivatized/extracted as described above. Time "zero" samples were processed in quadruplicate, all the other samples were analyzed in duplicate.

The inhibitory potency of SNACET, GSNO, CysNO and HcysNO was determined by measuring DDAH activity in rat

liver homogenate (100 mg/ml) in the presence of PIM (10 vol.%) and varying *S*-nitrosothiols concentration (0–1000 μM) as described above using ADMA at the fixed concentration of 100 μM and a fixed incubation time of 15 min. In these experiments *S*-nitrosothiol stock solutions of 10 mM each were prepared, which were further diluted with PBS to obtain appropriate dilutions. Incubations were performed in duplicate for each *S*-nitrosothiol concentration tested and in quadruplicate for incubations not containing ADMA and *S*-nitrosothiols.

2.3. Human and animal studies—ethics

The animal studies had been approved by the local supervisory committees for studies in animals (Hannover, Würzburg). In the pilot human study two authors of the present article (DT and DOS) served as healthy volunteers, thus no approval from the local Ethics committee of the Medical School of Hannover was necessary.

2.3.1. Animal studies

For method development, validation and application to study enzyme kinetics, hepatic DDAH activity a single liver (about 10.7 g) of a 5-month old, healthy, female Wistar rat (weighing about 320 g) was used. The rat was anaesthetized with CO_2 and killed by throat section. The liver was taken out immediately, washed with calcium- and magnesium-free PBS and cut in small pieces which were frozen in liquid nitrogen and finally stored at −80 °C until use.

The GC–MS method was applied to evaluate hepatic DDAH activity in diabetic ($n = 4$) and non-diabetic rats ($n = 4$). Diabetes was induced by a single intravenous injection of streptozotocin (50 mg/kg) in male Wistar rats (weighing 180–200 g) obtained from Harlan-Winkelmann (Borchen, Germany) as described [14]. After 12 weeks hyperglycemia was confirmed by blood glucose monitoring system in the morning after an overnight fasting period (Ascensia EliteTM, Bayer, Germany). Only streptozotocin-treated rats with blood glucose levels >300 mg/dl were included in the study.

2.3.2. Human study

In previous work we showed that the diurnal excretion of DMA and ADMA is quite constant in three healthy humans under basal conditions and does not change upon oral administration of the diuretics acetazolamide [3]. In a pilot study we investigated in the present work the effect of orally administered SNACET on the urinary excretion rate of DMA in two healthy volunteers. For this, urine was collected before and up to 7 h after oral administration of S^{15}NACET at a dose of 1 μmol S^{15}NACET/kg bodyweight. In urine samples we measured DMA by GC–MS [3], nitrite and nitrate by GC–MS without external addition of ^{15}N-labelled nitrite and nitrate [15], and creatinine by HPLC [16]. S^{15}NACET was freshly prepared by mixing 10 ml of an aqueous 10-mM solution of NACET with 10 ml of a 10-mM solution of ^{15}N-labelled nitrite (sodium salt) and 200 μl of 5 M HCl. Immediately prior to administration this solution was diluted with drinking water.

The urinary excretion rate of DMA and S^{15}NACET-derived ^{15}N-labelled nitrite and nitrate were corrected for creatinine excretion. In the case of DMA, creatinine-corrected excretion rate was expressed as μmol of DMA per mmol of creatinine. In the case of ^{15}N-labelled nitrite and nitrate, the peak area ratios (R) of m/z 47 ([^{15}N]nitrite) to m/z 46 ([^{14}N]nitrite) for nitrite and of m/z 63 ([^{15}N]nitrate) to m/z 62 ([^{14}N]nitrate) for nitrate were multiplied by 1000 (dimensionless) and divided by the respective creatinine concentration (in mM).

2.4. GC–MS conditions

GC–MS was performed on a ThermoElectron DSQ quadrupole mass spectrometer connected directly to a ThermoElectron Focus gas chromatograph and to an autosampler AS 3000 (ThermoElectron, Dreieich, Germany). A fused-silica capillary column Optima-17 (15 m × 0.25 mm i.d., 0.25-μm film thickness) from Macherey-Nagel (Düren, Germany) was used. Aliquots (1 μl) of the toluene extracts were injected in the splitless mode. The following oven temperature program was used with helium (constant flow of 1 ml/min) as the carrier gas: 1 min at 70 °C, then increased to 280 °C at a rate of 30 °C/min, and to 300 °C at a rate of 10 °C/min. Interface, injector and ion-source were kept at 260, 200 and 250 °C, respectively. Electron energy and electron current were set to 50 eV and 120 μA, respectively, for positive-ion chemical ionization (PICI) with methane (2.4 ml/min) as the reagent gas. Quantification by GC–MS in the PICI mode was performed by selected-ion monitoring (SIM) of the ions at m/z 240.15 for d_0-DMA and m/z 246.15 for d_6-DMA using a dwell-time of 50 ms for each ion and an electron multiplier voltage of 1.5 kV.

3. Results and discussion

3.1. Intra-assay precision and accuracy of the method for DMA quantification in rat liver homogenate

Data from the intra-assay validation experiment for the GC–MS method for DMA in rat liver homogenate are presented in Table 1. In the concentration range for added DMA (0–26 μM), recovery and imprecision ranged between 95–102% and 0.4–9.5%, respectively. Linear regression analysis between measured (y) and added (x) DMA concentration resulted in a straight line with the regression equation $y = 2.05 + 1.00x$, with $R = 0.99995$ and $P < 0.0001$. These results indicate that the present method is accurate and precise within the entire concentration range of DMA in rat liver homogenate suspensions. The DMA concentrations used in the validation experiment are relevant and were usually measured in liver homogenates after incubation with ADMA at final added concentrations of up to 100 μM (see below). Thus, quantitative determination of DMA as pentafluorobenzamide derivative by the present GC–MS method seems to proceed with the same accuracy and precision in rat liver homogenate, human plasma [2] and human urine [3].

Table 1
Intra-assay accuracy (recovery) and imprecision (RSD) of the GC–MS assay in a complete rat liver homogenate[a]

DMA added (μM)	DMA measured (μM) (mean ± SD, $n = 4$)	Recovery[b] (%)	Imprecision (%)
0	2.09 ± 0.2	N.A.	9.5
1	3.11 ± 0.13	101.5	4.2
2	4.06 ± 0.05	98.3	1.2
3	5.01 ± 0.44	97.2	8.8
4	6.11 ± 0.21	100.4	3.4
5	6.95 ± 0.14	97.1	2.0
6	7.82 ± 0.42	95.4	5.4
8	9.93 ± 0.26	97.9	2.6
10	12.1 ± 0.25	100.1	2.1
14	16.1 ± 0.06	100.0	0.4
18	20.4 ± 0.64	101.7	3.1
22	23.2 ± 1.05	95.9	4.5
26	28.3 ± 0.61	100.8	2.2

[a] No ADMA was externally added.
[b] Recovery (%) is calculated as: [(DMA measured − DMA basal):DMA added] × 100. N.A., not applicable.

The internal standard d_6-DMA was used in all experiments of the present work at a fixed concentration of 10 μM, like in human plasma and serum [2], in order to cover the whole DMA concentration range. The relatively constant peak area of the ion with *m/z* 246 in liver homogenates for incubation times of up to 120 min (RSD of the order of 20% for the peak area) suggests that d_6-DMA is stable and does not undergo metabolism by hepatic enzymes under these conditions. It has been shown that the hydrolysis of ADMA by DDAH is irreversible [9]; this excludes the possibility of d_6-DMA and DMA loss by re-conversion to d_6-ADMA and ADMA, respectively.

3.2. Characterization of the DDAH assay—Michaelis–Menten kinetics

Fig. 2 shows that formation of DMA in rat liver homogenate is dependent upon incubation time and concentration of externally added ADMA (0–100 μM). Without external addition of ADMA a relatively constant concentration of about 3 μM for DMA was measured in the liver homogenate in the whole observation time, indicating that ADMA formation from hepatic tissue is inhibited by PIM. Upon addition of ADMA, DMA concentration increased with incubation time and ADMA concentration to reach final values of up to 27 μM. Fig. 2 shows that after 30 min of incubation the kinetics of DMA formation becomes slower. The reason for this drop could be oxidation of the cysteine moiety in the active-site of DDAH rather than consumption of ADMA. On the basis of this experiment, an incubation time of 15 min and added ADMA concentrations in the range investigated were considered appropriate for determining DDAH activity under the conditions used in the present study. Partial chromatograms from the quantitative determination of DMA in liver homogenates incubated with various ADMA concentrations are shown in Fig. 3.

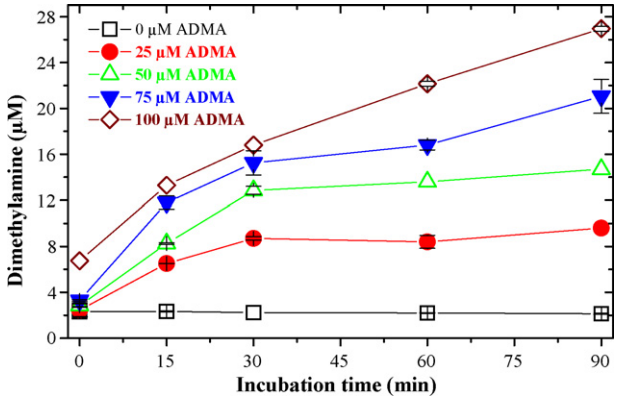

Fig. 2. DMA concentrations measured by GC–MS in rat liver homogenates in PBS in the presence of the protease inhibitor mixture (PIM, 10 vol.%) after incubation at 37 °C for the indicated time with added ADMA concentrations of 0, 25, 50, 75, and 100 μM. The liver tissue content was 83 mg/ml in all incubates. See also Fig. 3.

In these experiments the highest DMA formation rate was obtained by adding ADMA at a final concentration of 100 μM (Fig. 2). In this case a considerable amount of DMA was formed a few seconds after ADMA addition, i.e. until subjection of the sample to derivatization/extraction. The methodological difficulty in obtaining a true "time zero" value could be overcome by stopping the reaction either by using a DDAH inhibitor or by inactivating the enzyme. Very recently the inhibition of DDAH activity by two synthetic guanidine-substituted compounds has been reported [11]; however, these inhibitors are not commercially available so far [11]. L-NMMA could also be used to stop the DDAH-catalyzed hydrolysis of ADMA to DMA and L-citrulline, however, because L-NMMA itself is a substrate for DDAH [9] and a very weak inhibitor of DDAH, use of L-NMMA may be ineffective even at the very high concentration of 500 μM [8]. Alternatively, organic solvents, acids or bases or in particular SH-specific reagents such as *p*-chloromercuribenzoate or $HgCl_2$ [9] can also be used to stop the enzymatic reaction (see below).

Fig. 4 shows that the formation rate of DMA is dependent upon the liver tissue content up to 100 mg/ml in the incubation mixtures. A closer look reveals that DMA formation rate was smaller in the range 0–25 mg/ml as compared to that in the range 25–100 mg/ml. For measuring DDAH activity we decided to use liver tissue contents in the range 50–100 mg/ml because DMA concentrations formed at higher protein concentrations are clearly above the basal DMA concentrations unlike at lower protein concentrations (see Fig. 4). Nevertheless, linear regression analysis between DMA formation rate (y) and liver tissue content (x) in the whole range resulted in a straight line ($R = 0.99631$) with the regression equation $y = -2 + 0.53x$. On the basis of this data, the mean specific DDAH activity is calculated as 5.3 pmol DMA/min mg rat liver. Considering that in average 13.3 mg rat liver/ml corresponds to 1 mg protein/ml (see above), the mean specific DDAH activity is estimated to 70 pmol DMA/min mg protein. DDAH activity (measured as ADMA decay rate) of the same order of magnitude in liver and kidney tissue of mice and rats has been reported by other groups [7–9].

Fig. 5 shows that the DDAH-catalyzed hydrolysis of ADMA to DMA followed a hyperbolic function (Fig. 5A) with a linear Lineweaver–Burk plot between the reciprocal specific activ-

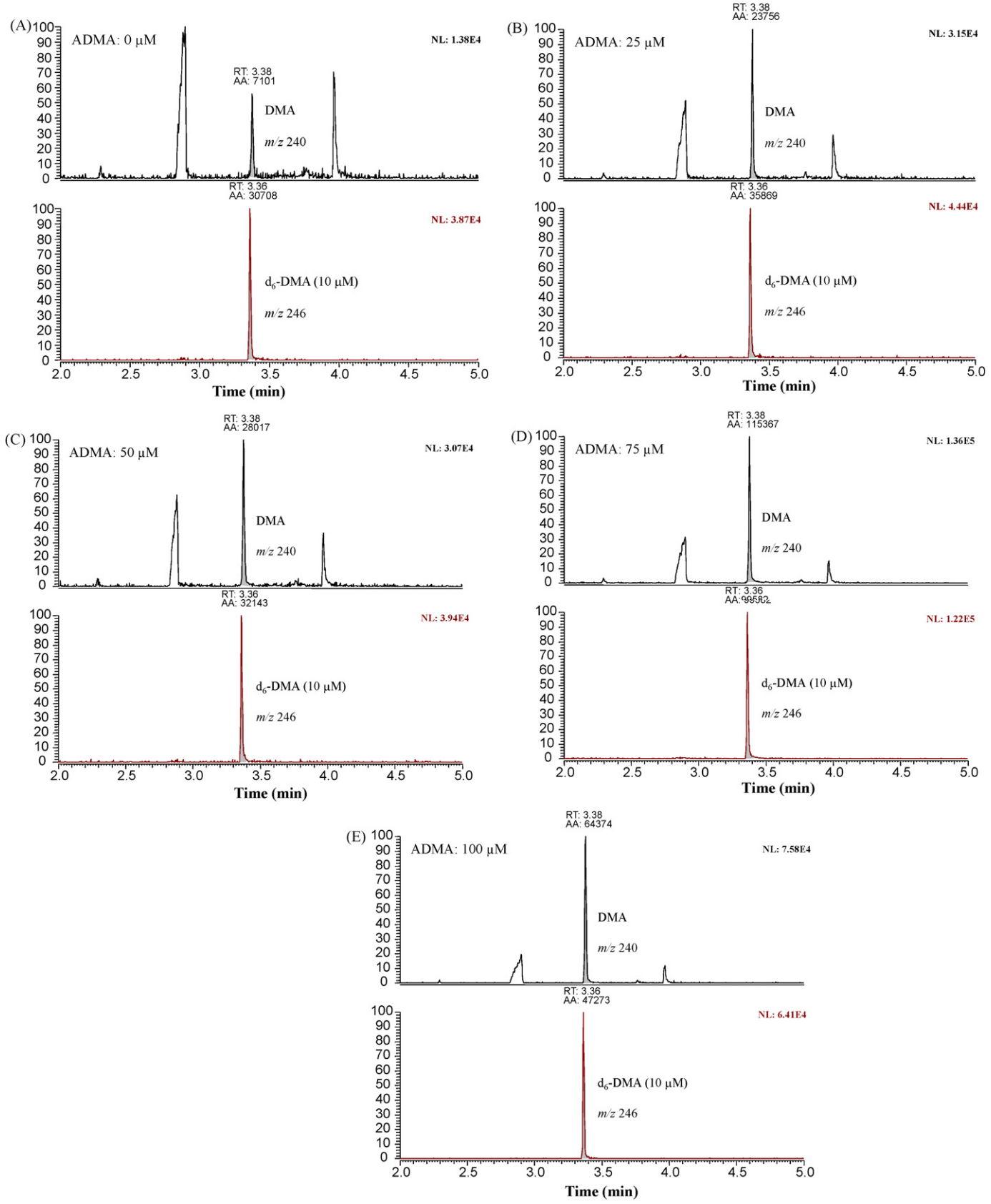

Fig. 3. Partial GC–MS chromatograms from the quantitative determination of DMA in rat liver homogenates in PBS incubated with various ADMA concentrations (0, 25, 50, 75, 100 μM) each for 15 min at 37 °C in the presence of the protease inhibitor mixture (PIM, 10 vol.%). The liver tissue content was 83 mg/ml in all incubates. Selected-ion monitoring of *m/z* 240.15 for endogenous and ADMA-derived DMA (d_0-DMA, upper tracing) and *m/z* 246.15 for the internal standard (d_6-DMA, lower tracing) which was added to the homogenate at a final concentration of 10 μM. See also Fig. 2.

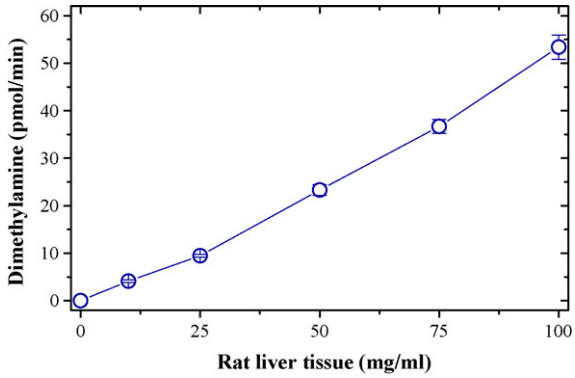

Fig. 4. Dependence of DMA formation rate in rat liver homogenates in PBS upon the tissue content in the homogenate suspensions in the presence of the protease inhibitor mixture (PIM, 10 vol.%). The incubation (at 37 °C) time and ADMA added concentration were each 15 min and 40 μM, respectively. The DMA concentration measured in the absence of liver was subtracted from each DMA concentration measured in the presence of liver tissue. From the difference the amount of DMA present in 0.1 ml assay volume was calculated and divided by 15 min to obtain the formation rate of DMA in pmol/min. *Note*: a value of 13.3 mg rat liver/ml corresponds to about 1 mg protein/ml. The DMA concentrations measured were 4.9, 5.5, 6.3, 8.4, 10.4 and 12.9 μM, respectively.

ity ($1/V$, y) and the reciprocal ADMA concentration ($1/C$, x) (Fig. 5B). From the regression equation $y = 0.08 + 4.78x$, K_M and V_{max} were determined to be 59.8 μM for ADMA and 12.5 pmol DMA/min mg liver (corresponding to 166 pmol DMA/min mg protein), respectively. K_M and V_{max} values for rat kidney DDAH have been reported to be 180 μM ADMA and 280 pmol/min mg protein, respectively, by measuring formation of ^{14}C-labelled L-citrulline from ^{14}C-labelled ADMA [9].

The pH value and temperature at which purified DDAH showed maximum activity has been reported to be 6.5 and 55 °C, respectively, when ADMA was used as the substrate [9]. It has also been reported that DDAH was stable between pH 5.0 and 8.5 when incubated at 37 °C for 1 h. In the present work we performed all DDAH assays in PBS of pH 7.1 at 37 °C. The choice of the working pH value of 7.1 for our DDAH assay allowed us to use the same derivatization/extraction procedure previously shown to be optimum for DMA measurement in plasma or serum [2] without any further sample manipulation. Under the conditions of the present assay DDAH activity in rat liver homogenate seems to be less stable with incubation time (see Fig. 2).

In experiments performed at 7 different days within a period of about 3 months, DDAH activity was determined under identical experimental conditions including ADMA concentration (100 μM), protein liver content (about 100 mg/ml), incubation time (15 min) and presence of PIM (10 vol.%) using the liver of the same rat. In these experiments DDAH activity was determined to be 8.74 ± 0.62 pmol/min mg liver, i.e. with an inter-assay imprecision (RSD) of 7.1%.

3.3. Effect of sulfhydryl group-specific agents on DDAH activity in rat liver

It has been reported that the presence of the reduced form of a cysteine moiety in the active-site of DDAH, e.g. Cys-249 of *Pseudomonas aeruginosa*, is essential for DDAH activity [17,18]. This hypothesis is mainly based on the observation that in vitro incubation of recombinant DDAH proteins with the NO donor DEANONOate inhibited DDAH activity [18]. Because dithiothreitol (DTT) reversed the action of DEANONOate, it was assumed that the *S*-nitrosation of the SH group of Cys-249 in DDAH by this NO donor is the principle of the inhibitory action of NO and NO donors on DDAH [18]. This hypothesis is supported by pioneer findings showing that SH-blocking substances including mercuri-containing substances, i.e. $HgCl_2$ and *p*-chloromercuribenzoate, inhibited DDAH activity [9].

In the present study we addressed this particular issue and investigated the effects of two different SH-specific agents, i.e. $HgCl_2$, the synthetic *S*-nitrosothiol SNACET, and the putatively biological *S*-nitrosothiols GSNO, CysNO, HcysNO. Fig. 6 shows that $HgCl_2$ (at 130 μM following [9]) and SNACET (at 260 μM) inhibited DMA formation from externally added ADMA in rat liver homogenate.

Because of the potential usefulness of *S*-nitrosothiols as a therapeutic means to inhibit DDAH activity [17,18] we inves-

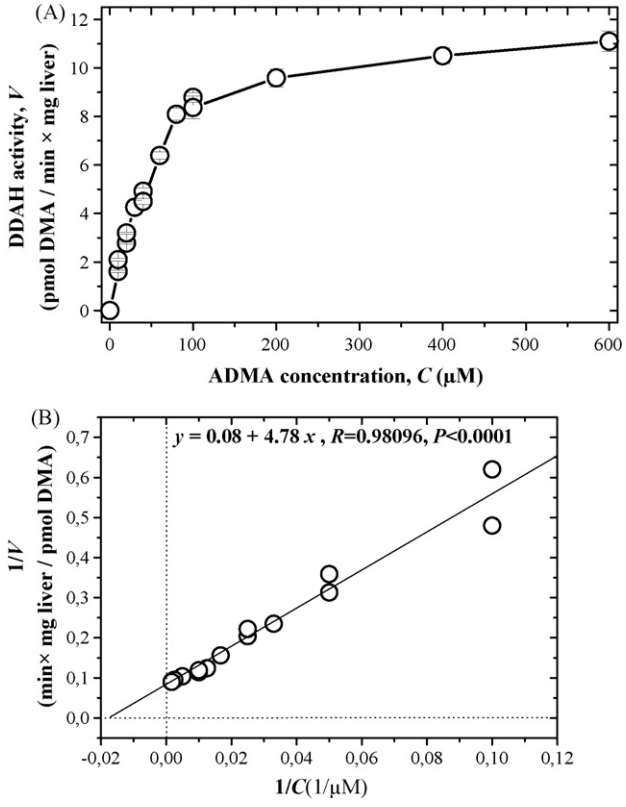

Fig. 5. Kinetics of DDAH-catalyzed hydrolysis of ADMA to DMA in rat liver homogenate in 100 mM PBS, pH 7.1, at 37 °C. (A) Direct plot. (B) Lineweaver–Burk plot. Liver tissue content and incubation time were 75 mg/ml and 15 min for all incubates, respectively. All incubates contained PIM (10 vol.%). The DDAH activity in the sample which had not been spiked with ADMA ($n = 9$) was subtracted from those measured in incubates being spiked with the indicated ADMA concentrations (each $n = 4$). This Figure was constructed with data generated from two experiments which were performed within an interval of about 3 months. ADMA final concentrations were 0, 10, 20, 30, 40, 60, 80 and 100 μM in the first experiment, and 0, 10, 20, 40, 100, 200, 400 and 600 μM in the second experiment. *Note*: a value of 13.3 mg rat liver/ml corresponds to about 1 mg protein/ml.

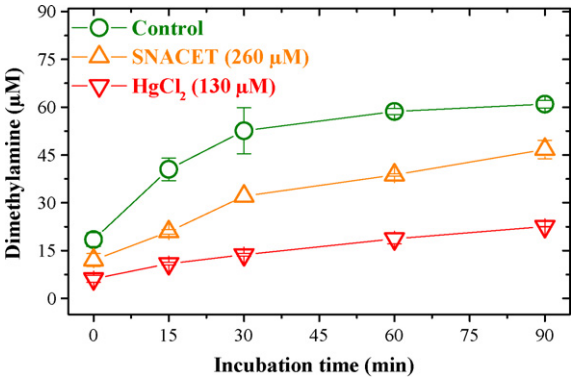

Fig. 6. Effect of the SH-blocking agents *S*-nitroso-*N*-acetylcysteine ethyl ester (SNACET, 260 μM) and $HgCl_2$ (130 μM) on the DDAH-catalyzed formation of DMA from externally added ADMA (91 μM) in rat liver homogenates (about 100 mg liver/ml) in 100 mM PBS, pH 7.1, at 37 °C. All incubates contained PIM (10 vol.%). For more detail see the text.

tigated the effect of the synthetic SNACET and putatively endogenous *S*-nitrosothiols on DDAH activity in more detail. Fig. 7 shows that SNACET, even at the very high concentration of 1000 μM, did not completely inhibit DDAH activity. From this experiment the half-maximal inhibitory concentration (IC_{50}) of SNACET for rat liver DDAH is estimated to be about 500 μM. GSNO, CysNO and HcysNO were also found to inhibit rat liver DDAH activity in a concentration-dependent manner, with estimated IC_{50} values being 1000 μM, 700 μM, and 300 μM, respectively. In consideration of the considerably higher content of sulfhydryl groups in tissue and the rapid and extensive in vivo metabolism of *S*-nitrosothiols including SNACET (see below) we may assume that in vivo the IC_{50} values of these *S*-nitrosothiols may be even considerably higher.

Previous work showing almost complete inhibition of (presumably) rat kidney DDAH by 100 μM of $HgCl_2$ [9] and the present study suggest that at a molar basis $HgCl_2$ is a stronger inhibitor of DDAH than the *S*-nitrosothiols tested. In addition to differences in inhibitory potency of $HgCl_2$ and *S*-nitrosothiols, we may reasonably assume that there will also be differences with respect to the type of inhibition.

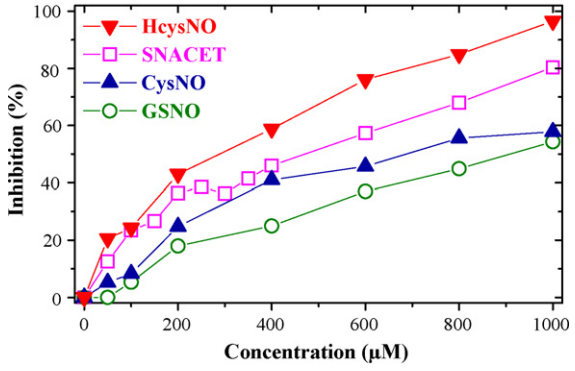

Fig. 7. Extent of inhibition of DDAH activity by different *S*-nitrosothiols at varying concentrations as indicated. DDAH activity was determined by incubating ADMA (100 μM) for 15 min in rat liver homogenates (100 mg liver/ml) in 100 mM PBS, pH 7.1, at 37 °C. All incubates contained PIM (10 vol.%). For more detail see the text.

Thus, $HgCl_2$ would act as an irreversible inhibitor because it would react covalently with the SH-group of Cys-249 in *Pseudomonas aeruginosa* (Eq. (1)), unlike SNACET and other *S*-nitrosothiols (R–S–NO) which would inhibit DDAH rather reversibly because *S*-transnitrosylation reactions are equilibrium reactions (Eq. (2)) [19,20], with equilibrium constants being close to the unity [19]. Eventually, Fig. 6 suggests that $HgCl_2$ may be a useful reagent to stop DDAH activity in DDAH assays.

$$HgCl_2 + DDAH\text{–}Cys^{249}\text{–}SH \rightarrow DDAH\text{–}Cys^{249}\text{–}S\text{–}HgCl + HCl \quad (1)$$

$$R\text{–}S\text{–}NO + DDAH\text{–}Cys^{249}\text{–}SH \leftrightarrow R\text{–}S\text{–}H + DDAH\text{–}Cys^{249}\text{–}S\text{–}NO \quad (2)$$

Our present findings showing GSNO and CysNO as inhibitors of rat liver DDAH activity contradict the results of a recent study by Braun et al. [21], in which GSNO and CysNO (in the range 0–300 μM) turned out not to be inhibitors of bovine kidney DDAH and isolated DDAH-1 activity as measured by L-citrulline formation from L-NMMA. By contrast, in this [21] and in previous work [22] of the same group, HcysNO was found to inhibit DDAH activity with an IC_{50} value of about 75 μM [21], which is considerably lower than the IC_{50} value of 300 μM found in the present study. To overcome the obvious discrepancy between HcySNO and GSNO or CysNO, this group has suggested that HcysNO reacts covalently with the SH-group of the cysteine moiety in the active-site of DDAH to form an *N*-thiosulfoximide [21] (see Eq. (3)), unlike GSNO and CysNO. Formation of this derivative was reportedly demonstrated by Nano-ESI-MS using HcysNO at the very high concentration of 500 μM [21,22]. However, the extent of the *N*-thiosulfoximidation of DDAH by HcysNO has not been reported. Braun et al. reported that HcysNO inhibited DDAH activity by this mechanism with IC_{50} of 75 μM even in the presence of mM-concentrations of GSH, and assumed that HcysNO was "stable" in these conditions [21]. However, this assumption has not been substantiated by the authors, which, moreover, contradicts the thermodynamics and kinetics of the Hcys/HCysNO/GSH/GSNO system. Considering an equilibrium constant (K_{eq}) of 2.09 [19] for this binary *S*-nitrosothiol/thiol system (Eq. (4)) and a Hcys concentration in the low nM-range, it can be estimated that addition of HcysNO (0–300 μM) to DDAH incubates containing GSH at about 2 mM [21] would shift this reversible *S*-transnitrosylation reaction (Eq. (4)) at the expense of HcysNO. Further investigations are needed to clarify these discrepancies and the putatively divergent behavior of HcysNO from that of GSNO and CysNO. Also, it would be of interest to investigate whether the synthetic SNACET can inhibit DDAH activity by an *N*-thiosulfoximidation-based mechanism.

$$Hcys\text{–}S\text{–}NO + DDAH\text{–}Cys\text{–}SH \rightarrow DDAH\text{–}Cys\text{–}S(=O)\text{–}NH\text{–}S\text{–}Hcys \quad (3)$$

$$Hcys\text{–}SH + GSNO \leftrightarrow Hcys\text{–}S\text{–}NO + GSH \quad (4)$$

3.4. Effect of S^{15}NACET on urinary excretion of DMA in healthy volunteers

Fig. 8 shows that oral administration of S^{15}NACET to two healthy volunteers resulted in immediate increases in the creatinine-corrected peak area ratios (*R*) of [^{15}N]nitrite and [^{15}N]nitrate suggesting rapid absorption of S^{15}NACET and conversion of its *S*-[^{15}N]nitroso group to [^{15}N]nitrite and [^{15}N]nitrate which are eventually excreted in the urine. Unlike in vitro inhibition of DDAH activity by SNACET (Figs. 6 and 7), Fig. 8 shows that in vivo DMA excretion rate was not reduced after administration of S^{15}NACET. Moreover, it seems that DMA excretion increased temporally upon S^{15}NACET administration. Although very preliminary, these results are not supportive of the suggestion that *S*-nitrosation by higher oxides of NO and *S*-transnitrosylation by *S*-nitrosothiols of DDAH may represent a significant mechanism by which DDAH activity could be inhibited in vivo in humans under basal conditions or after treatment with NO, NO- and NO^+-donors including *S*-nitrosothiols. When investigating a potential role of endogenous *S*-nitrosothiols in the inhibition of DDAH activity, we should have in mind that putative endogenous *S*-nitrosothiols, such as GSNO, CysNO and HcysNO, occur in body fluids and tissue most likely at concentrations in the very low nM-range when ever [20]. Therefore, results obtained using extremely high concentrations of NO and NO donors [11], of *S*-nitrosothiols such as 500 μM of HcysNO [21,22], and of oxidants should be treated with extreme caution.

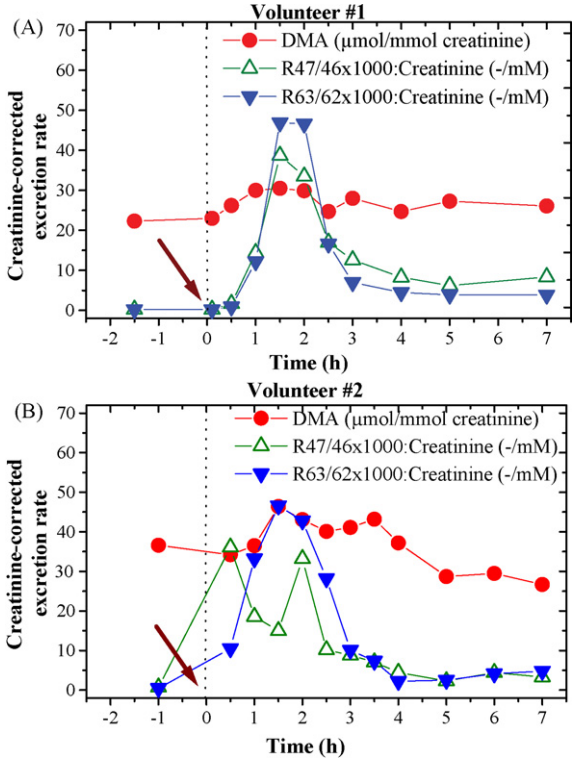

Fig. 8. Effect of orally administered *S*-[^{15}N]nitroso-*N*-acetylcysteine ethyl ester (S^{15}NACET; 1 μmol/kg) on creatinine-corrected excretion rate of dimethylamine (DMA) and ^{15}N-labelled nitrite and nitrate in two healthy volunteers. S^{15}NACET was taken in drinking water. The arrows indicate the time of administration which was set to time zero. R47/46 and R63/62 means peak area ratios of ^{15}N-labelled to ^{14}N-labelled nitrite and nitrate, respectively. For more details see the text.

In our opinion, the importance ascribed to *S*-nitrosylation and oxidative stress as physiological regulation mechanisms of mammalian DDAH isoforms [17,18,21,22] are overrated (see also below). Our restraint on the overstated significance of the regulatory mechanisms of *S*-nitrosylation and oxidative stress is supported by a recent study on *Pseudomonas aeruginosa* DDAH [23]. This study suggests that the neutral cysteine residue found in the resting state of this bacterial DDAH may be more resistant to inhibition by cellular oxidants and *S*-nitrosylation than would be the case if this enzyme used a pre-formed ion pair in its mechanism [23]. It is worth mentioning to point out that the reactivity of relevant cysteine moieties in proteins such as albumin and haemoglobin and presumably in enzymes may also differ from species to species [24]. Thus, potential organ- and species-specific differences in the reactivity of the cysteine moiety in the active-site of DDAH should be considered.

3.5. Hepatic DDAH activity in diabetic and non-diabetic rats

Table 2 shows that hepatic DDAH activity is statistically significantly ($P = 0.01$) higher in diabetic rats (293 pmol DMA/min mg protein) as compared to non-diabetic rats (167 pmol DMA/min mg protein). The DDAH activity measured in the four male non-diabetic Wistar rats is of the same order of that measured in the non-diabetic female Wistar rat (see above).

The L-arginine/NO pathway is impaired in streptozotocin-induced diabetes mellitus in rats [14,25]. Interestingly, in the study by Lin et al. [25] aortic DDAH activity (measured as the decrease in ADMA concentration) but not DDAH expression was found to be reduced in diabetic rats as compared to non-diabetic rats. Concerning DDAH activity, the results of the present study seem to contradict the results of the study by Lin et al. [25]. However, this contradiction may be due to differences in the animal models used including origin of DDAH, i.e. abdominal aorta, cultured vascular smooth muscle cells isolated from abdominal aortas and cultured human dermal microvascular endothelial cells [25] versus liver tissue (present study), as well as due to differences in diet, i.e. high-fat diet [25] versus normal chow (present study). Methodological differences in the DDAH assays used, e.g. measurement of DMA in the present study versus measurement of ADMA or L-citrulline [25], could also have contributed to the contradictory results; however, it should be noted that DDAH activity was of the same order in both studies.

The results of the present work suggest that streptozotocin-induced diabetes elevates hepatic DDAH activity in normal-fat diet male Wistar rats. The liver and the kidney are primarily responsible for the elimination of ADMA via the metabolic DDAH pathway [26]. It may be possible that the elevated circulating ADMA concentration found in various diseases, including diabetes [25], end-stage liver disease and coronary artery disease [3], leads to an elevation of DDAH activity in the liver (and presumably in the kidney) despite elevated oxidative stress which is

Table 2
DDAH activity in the liver of four non-diabetic and four diabetic male Wistar rats

Rat no.	Protein (mg/ml)		DMA measured (μM)				DDAH (pmol DMA/min mg protein)		
	Non-diabetic	Diabetic	Non-diabetic		Diabetic		Non-diabetic	Diabetic	ND/D[a] ratio
			−ADMA	+ADMA	−ADMA	+ADMA			
1/2	11.4	17.0	2.8 ± 0.1	26.1 ± 1.7	2.9 ± 0.1	52.2 ± 2.5	136	193	1.42
3/4	10.2	13.6	2.7 ± 0.2	28.2 ± 2.8	3.1 ± 0.2	66.5 ± 0.8	167	311	1.86
5/6	12.0	12.2	2.7 ± 0.2	36.9 ± 0.9	3.4 ± 0.1	53.5 ± 0.7	190	315	1.66
7/8	10.1	10.6	2.8 ± 0.2	29.3 ± 1.0	2.7 ± 0.3	58.6 ± 1.1	175	352	2.01
Mean ± SD	10.9 ± 0.9	13.4 ± 2.7	2.8 ± 0.1	30.1 ± 4.7	3.0 ± 0.3	57.7 ± 6.5	167 ± 23	293 ± 69[b]	1.7 ± 0.3

Note: A complete enzyme incubation mixture was used. In half of the samples no ADMA (−ADMA) was added; in the second half of the samples ADMA was added (+ADMA) at a final concentration of 100 μM. The liver content was 100 mg/ml in homogenates from all rats. Protein concentration was measured in each homogenate in duplicate by the method of Bradford [13]. The incubation time was each 15 min for all incubates. At least four incubates were used for each measurement in all rats. Statistical significance was tested using the unpaired *t*-test. A *P*-value of <0.05 was considered significant.

[a] ND, non-diabetic; D, diabetic; ND/D, ratio of DDAH specific activity in ND and D.

[b] $P = 0.01345$.

generally accepted to prevail in these diseases. The increased circulating ADMA concentration and the elevated DMA excretion rate measured in humans suffering from end-stage liver disease and coronary artery disease [3] are supportive of this idea.

4. Conclusions

The GC–MS assay reported in the present work is based on the measurement of DMA produced from DDAH-catalyzed hydrolysis of ADMA and is, therefore, more specific than DDAH assays measuring the decay of ADMA concentration or the formation of L-citrulline. The present study provides clear evidence that *S*-transnitrosylation of DDAH by endogenous and synthetic *S*-nitrosothiols is a principal inhibitory mechanism of DDAH activity in vitro. In consideration of the very potent biological activity of NO and *S*-nitrosothiols and their very weak inhibitory potency towards DDAH, our study does not suggest that *S*-transnitros(yl)ation or *N*-thiosulfoximidation of cysteine moieties in the active-site of DDAH may represent a rational design of DDAH inhibitors. Also, our work suggests that DDAH activity is not affected by diabetes-associated oxidative stress, at least in the liver of the rats investigated in the present study. The GC–MS methodology reported here and in previous work applies equally to the quantitative determination of circulating and excretory DMA in humans and to assess DDAH activity in tissue. This analytical feature should allow study effects of various treatments, including NO, NO- and nitrosyl (NO^+)-donors such as *S*-nitrosothiols, on DDAH activity in clinical and animal studies.

Acknowledgment

Kristine Chobanyan is recipient of a grant (A/06/08992) from the DAAD (Deutscher Akademischer Austauschdienst).

References

[1] T. Teerlink, M.W.T. Hennekes, C. Mulder, H.F.H. Brulez, J. Chromatogr. B 691 (1997) 269.
[2] K. Chobanyan, A. Mitschke, F.-M. Gutzki, D.O. Stichtenoth, D. Tsikas, J. Chromatogr. B 851 (2007) 240.
[3] D. Tsikas, T. Thum, T. Becker, V.V. Pham, K. Chobanyan, A. Mitschke, B. Beckmann, F.-M. Gutzki, J. Bauersachs, D.O. Stichtenoth, J. Chromatogr. B 851 (2007) 229.
[4] J.M. Leiper, P. Vallance, Eur. J. Clin. Pharmacol. 62 (2006) 33.
[5] D. Tsikas, R.H. Böger, J. Sandmann, S.M. Bode-Böger, J.C. Frölich, FEBS Lett. 478 (2000) 1.
[6] V. Achan, M. Broadhead, M. Malaki, G.S.J. Whitley, J. Leiper, R. MacAllister, P. Vallance, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1455.
[7] S. Nonaka, M. Tsunoda, C. Aoyama, T. Funatsu, J. Chromatogr. B 843 (2006) 170.
[8] R. Maas, J. Tan-Andreesen, E. Schwedhelm, F. Schulze, R.H. Böger, J. Chromatogr. B 851 (2007) 220.
[9] T. Ogawa, M. Kimoto, K. Sasaoka, J. Biol. Chem. 264 (1989) 10205.
[10] F.I. Arrigoni, P. Vallance, S.G. Haworth, J.M. Leiper, Circulation 107 (2003) 1195.
[11] J. Leiper, M. Nandi, B. Torondel, J. Murray-Rust, M. Malaki, B. O'Hara, S. Rossiter, S. Anthony, M. Madhani, D. Selwood, C. Smith, B. Wojciak-Stothard, A. Rudiger, R. Stidwill, N.Q. McDonald, P. Vallance, Nat. Med. 13 (2007) 198.
[12] K. Tewes, Ph.D. thesis, Hannover Medical School, 2000.
[13] M.M. Bradford, J. Biol. Chem. 72 (1976) 248.
[14] T. Thum, D. Fraccarollo, M. Schultheiss, S. Froese, P. Galuppo, J.D. Widder, D. Tsikas, G. Ertl, J. Bauersachs, Diabetes 56 (2007) 666.
[15] D. Tsikas, Anal. Chem. 72 (2000) 4064.
[16] D. Tsikas, A. Wolf, J.C. Frölich, Clin. Chem. 50 (2004) 201.
[17] J. Murray-Rust, J.M. Leiper, M. McAlister, J. Phelan, S. Tilley, J. Santa Maria, P. Vallance, N. McDonald, Nat. Struct. Biol. 8 (2001) 679.
[18] J. Leiper, J. Murray-Rust, N. McDonald, P. Vallance, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 13527.
[19] D. Tsikas, J. Sandmann, S. Rossa, F.-M. Gutzki, J.C. Frölich, Anal. Biochem. 270 (1999) 231.
[20] D. Giustarini, A. Milzani, I. Dalle-Donne, R. Rossi, J. Chromatogr. B 851 (2007) 124.
[21] O. Braun, M. Knipp, S. Chesnov, M. Vasák, Protein Sci. 16 (2007) 1522.
[22] M. Knipp, O. Braun, M. Vasák, J. Am. Chem. Soc. 127 (2005) 2372.
[23] E.M. Stone, A.L. Costello, D.L. Tierney, W. Fast, Biochemistry 45 (2006) 5618.
[24] D. Giustarini, A. Milzani, R. Colombo, I. Dalle-Donne, R. Rossi, Trends Pharmacol. Sci. 25 (2004) 311.
[25] K.Y. Lin, A. Ito, T. Asagami, P.S. Tsao, S. Adimoolam, M. Kimoto, H. Tsuji, G.M. Reaven, J.P. Cooke, Circulation 106 (2002) 987.
[26] M.P.C. Siroen, M.C. Warlé, T. Teerlink, R.J. Nijveldt, E.J. Kuipers, H.J. Metselaar, H.W. Tilanus, D.J. Kuik, J.R.M. van der Sijp, S. Meijer, B. van der Hoven, P.A.M. Leeuwen, Liver Transpl. 10 (2004) 1524.