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A stable-isotope based technique for the determination of dimethylarginine dimethylaminohydrolase (DDAH) activity in mouse tissue ☆

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Abstract

The enzyme dimethylarginine dimethylaminohydrolase (DDAH) is responsible for the hydrolysis of asymmetric dimethylarginine (ADMA) to L-citrulline and dimethylamine. DDAH is currently investigated as a promising target for therapeutic interventions, as ADMA has been found to be elevated in cardiovascular disease. In many tissues continuous endogenous formation of ADMA and L-citrulline poses considerable limitations to the presently used assays for DDAH activity, which are commonly based on the measurement of ADMA or L-citrulline. We therefore developed a stable-isotope-based assay suitable for 96-well plates to determine DDAH activity. Using deuterium-labeled ADMA ([$^{2}H_{6}$]-ADMA) as substrate and double stable-isotope labeled ADMA ([$^{13}C_{5}$-$^{2}H_{6}$]-ADMA) as internal standard we were able to simultaneously determine formation and metabolism of ADMA in renal and liver tissue of mice by LC–tandem MS. Endogenous formation of ADMA could largely be abolished by addition of protease inhibitors, while metabolism of [$^{2}H_{6}$]-ADMA was not significantly altered. The intra-assay coefficient of variation for the determination of endogenous ADMA and [$^{2}H_{6}$]-ADMA was 2.4% and 4.8% in renal and liver tissue, respectively. The inter-assay coefficient of variation for DDAH activity based on degradation of [$^{2}H_{6}$]-ADMA determined in separate samples from the same organs was determined to be 8.9% and 10% for mouse kidney and liver, respectively. The present DDAH activity assay allows for the first time to simultaneously determine DDAH activity and endogenous formation of ADMA, SDMA, and L-arginine in tissue.
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Keywords: L-Arginine; ADMA; SDMA; Assay; Stable isotopes; Mass spectrometry

1. Introduction

Asymmetric dimethylarginine (ADMA), an endogenously formed nitric oxide synthase (NOS) inhibitor, has been identified as a new risk marker and potential player in cardiovascular disease and cancer [1–3]. The enzyme dimethylarginine dimethylaminohydrolase (DDAH), which is primarily responsible for ADMA degradation (Fig. 1), is currently investigated as a promising target for therapeutic interventions [4–6]. Transgenic and knockout animal models with altered DDAH activity are being developed to study drug effects on DDAH activity in vivo in animals and in vitro in tissue samples [7,8].

For the initial characterization of the purified DDAH enzyme ^{14}C-radio labeled ADMA was used [9]. In further experiments N^{G}-monomethylarginine (L-NMMA) was used as a DDAH substrate instead of ADMA [10]. In recent years, unlabeled ADMA has become a more frequently used substrate, and estimates of DDAH activity are often based on the degradation of unlabeled ADMA added to the sample [11,12], or on formation of the reaction product L-citrulline [13]. So far, validation data are only available for the assay based on degradation of unlabeled ADMA [11,12].

In vivo as well as in vitro in tissue samples formation and degradation of ADMA, L-arginine and L-citrulline occur in parallel (Fig. 1), leaving room for considerable interference when decline in ADMA concentration or formation of L-citrulline are used to calculate DDAH activity [14,15]. The methods

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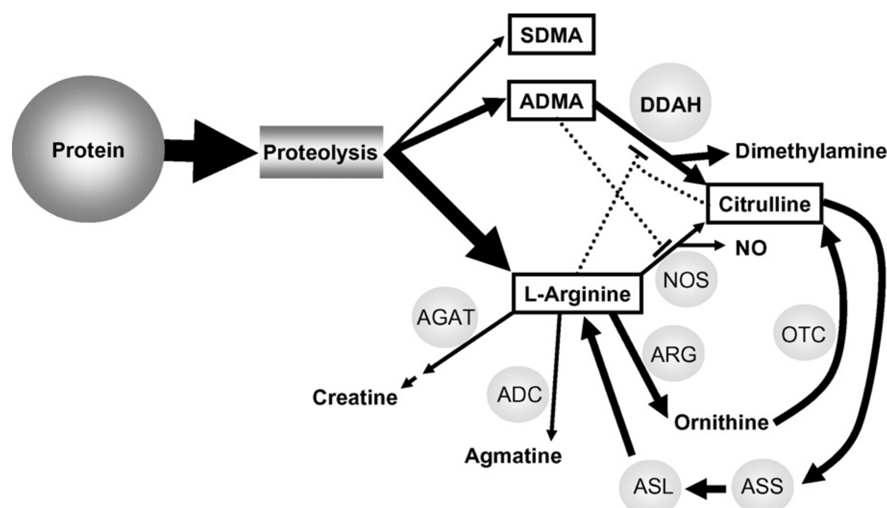

Fig. 1. Major pathways for formation and metabolism of ADMA, SDMA, and L-arginine. The extent and relative contributions of these pathways may vary from species to species and tissue to tissue. Abbreviations: ADC, arginine decarboxylase; AGAT, arginine–glycine amidinotransferase; ARG, arginase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; DDAH, dimethylarginine dimethylaminohydrolase; NOS, nitric oxide synthase; OTC, carnithine transcarbamylase.

presently available do not allow to simultaneously assess all these processes.

We therefore developed and validated a stable-isotope based method to simultaneously determine formation and degradation of ADMA and its analogues in a 96-well format assay.

2. Experimental

2.1. Materials and chemicals

L-Arginine hydrochloride reference standard was purchased from US Pharmacy (Rockvill, MD, USA). ADMA dihydrochloride (purity >99%) was from Sigma–Aldrich (Steinheim, Germany) and SDMA dihydrochloride (purity >99%) from Calbiochem (Schwalbach, Germany). Aqueous stock solutions of L-arginine, ADMA, and SDMA were made by weighing authentic material supplied by the manufacturer. L-[$^{2}H_{7}$]-Arginine hydrochloride ([2,3,3,4,4,5,5-L-$^{2}H_{7}$]-arginine, 98 atom% ^{2}H), L-[*guanidino*-$^{15}N_{2}$]-arginine hydrochloride (99 atom% ^{15}N); [1,1,1,1′,1′,1′-$^{2}H_{6}$]-dimethylamine (98 atom% ^{2}H); [1,2,3,4,5-$^{13}C_{5}$]-ornithine.HCl (99 atom% ^{13}C) and [1,1,1,1′,1′,1′-$^{2}H_{6}$]-ornithine.HCl (98 atom% ^{2}H) were purchased from Euriso-top (Saint-Aubin, France). The protease inhibitor mix (aprotinin 0.08 mM, AEBSF 104 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM, Sigma, Germany) was purchased from Sigma–Aldrich.

2.2. Chemical synthesis of stable-isotope labeled ADMA analogues

[$^{2}H_{6}$]-ADMA (i.e. [3,3,4,4,5,5-$^{2}H_{6}$]-ADMA) was prepared as previously described [16]. In brief, bromcyan-agarose (0.4 g) was suspended in 10 ml of 1 mM HCl in methanol and incubated for 30 min at room temperature. After incubation, the mixture was filtered and washed five times with 5 ml of 1 mM HCl and 5 ml of water. To prepare a copper–ornithine-complex, 0.125 g [$^{2}H_{6}$]-ornithine·HCl was dissolved in 1.25 ml of water. Small amounts of $CuCO_3$ were added until a blue colour was obtained. After filtration of the solution the filtrate was brought to pH 10 with 10 M KOH. The washed bromcyan-agarose was suspended in 1.25 ml of the copper–ornithine-complex and was shaken over night at 4 °C. The following day the suspension was filtered and washed one time with 1 M HCl and ten times with 10 ml water. The [$^{2}H_{6}$]-ornithine–bromcyan-agarose was then suspended in a 20 wt.% solution of dimethylamine in water and stirred for 24 h at 50 °C. The suspension was filtered and washed two times with 10 ml of water. The filtrate together with the washings was concentrated to dryness in vacuum to remove any remaining (volatile) dimethylamine. The residue was solved in water again and concentrated to dryness in vacuum for a second time. The residue was dissolved in water to yield a stock solution, which was stored at −20 °C.

Synthesis of [1,1,1,1′,1′,1′-$^{2}H_{6}$-dimethylamino-1,2,3,4,5-$^{13}C_{5}$]-arginine (i.e. [$^{13}C_{5}$-$^{2}H_{6}$]-ADMA) followed the same steps as described for [$^{2}H_{6}$]-ADMA with the following modifications: [$^{13}C_{5}$]-ornithine·HCl was used instead of [$^{2}H_{6}$]-ornithine·HCl to prepare the copper–ornithine complex, and the [$^{13}C_{5}$]-ornithine–bromcyan-agarose was suspended in a 20 wt.% solution of [$^{2}H_{6}$]-dimethylamine (prepared from [$^{2}H_{6}$]-dimethylamine gas). Purity and identity of [$^{2}H_{6}$]-ADMA and [$^{13}C_{5}$-$^{2}H_{6}$]-ADMA was assessed by HPLC (Fig. 2) and LC–MS (Fig. 3). In brief, fluorescence (RF2000 fluorescence detector, Dionex, Germering, Gernamy) was detected as previously described [17] after solid-phase extraction (SPE) with carboxylic acid (CBA) cartridges, online *o*-phthaldehyde (OPA) derivatisation, and separation on a phenyl column (250 mm × 4.6 mm i.d.) from Macherey-Nagel (Düren, Germany), at excitation and emission wavelengths of 340 and 453 nm, respectively. Mass spectra were generated by direct injection without prior chromatography.

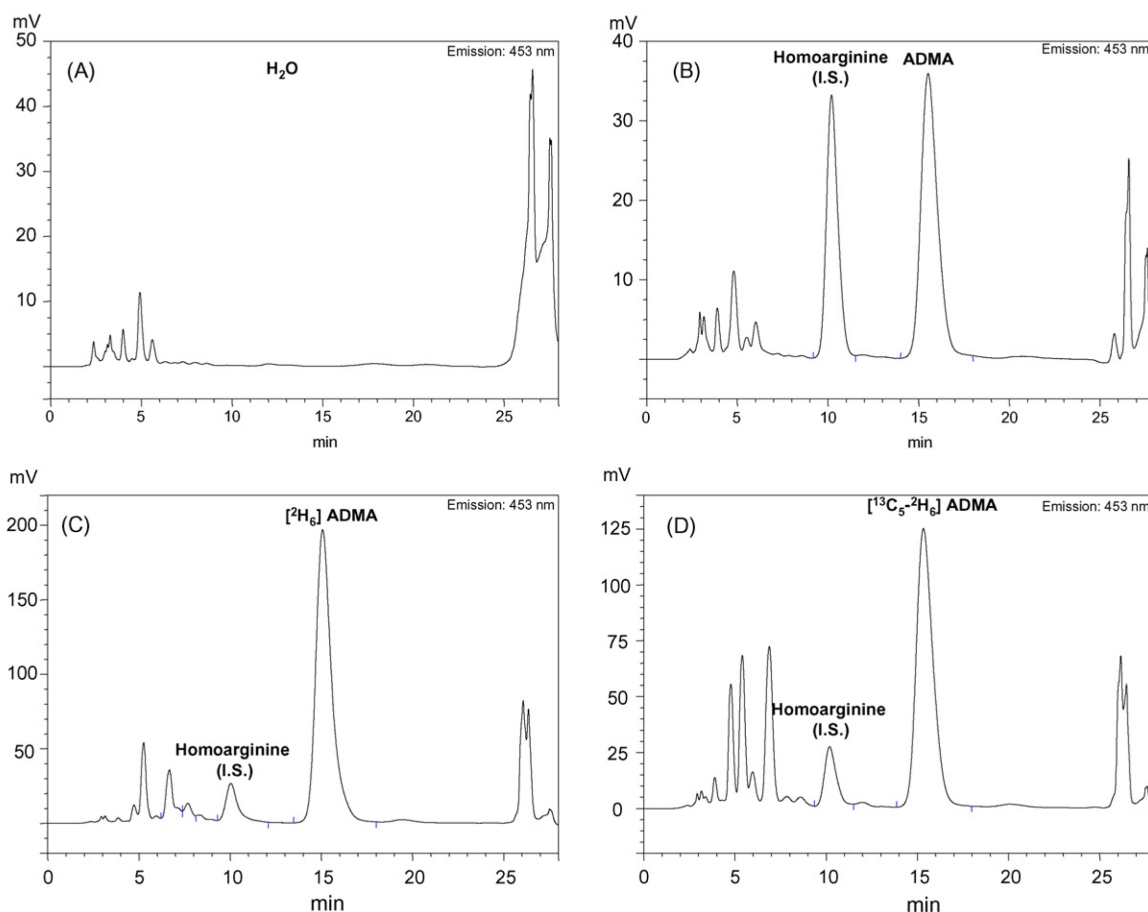

Fig. 2. HPLC chromatograms from the analysis of H_2O (A), ADMA (B), and newly synthesized [3,3,4,4,5,5-$^2H_6$]-ADMA (C) and [1,1,1,1′,1′,1′-$^2H_6$-dimethylamino-1,2,3,4,5-$^{13}C_5$]-arginine ([$^{13}C_5$-$^2H_6$]-ADMA]) (D). Fluorescence of OPA derivatives was detected after SPE with CBA cartridges, pre-column OPA derivatization, and separation on a phenyl column as described elsewhere [17].

2.3. DDAH activity assay

Kidney tissue (prepared from whole organs, stored at −20 °C) of C57BL6 mice was chosen as it is known to contain both isoforms of DDAH (DDAH1 and DDAH2) [18], as well as other major enzymes involved in L-argininine and L-citrulline metabolism [19]. All animal studies were approved by the local ethical review committee, and were carried out in accordance with German government regulations and NIH guidelines on the care and welfare of laboratory animals.

Initial explorative experiments to determine endogenous ADMA and L-arginine formation were performed using 10–100 mg frozen tissue samples homogenized on ice in 1:4–1:8 (w/v) in PBS buffer (NaCl 137 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM, with pH adjusted to 6.4 by HCl) to achieve final protein concentrations of 5–30 g/l. For the explorative experiments a standard protease inhibitor mix was added at a final dilution of 1:20 (v/v) when required to demonstrate the impact of proteolysis. Aliquots of 100 μl were incubated in 96-well plates for 5–60 min at 37 °C using 1–100 μM of [$^2H_6$]-ADMA as substrate. For determination of ADMA, L-arginine and SDMA at specific time points 10 μl aliquots were taken from the reaction wells and the reaction was stopped on ice by addition of 100 μl of methanol which contained [$^{13}C_5$ -$^2H_6$]-ADMA and [$^2H_7$]-L-arginine as internal standards for ADMA, [$^2H_6$]-ADMA and SDMA, and for L-arginine, respectively, corresponding to final concentrations of [$^{13}C_5$-$^2H_6$]-ADMA and [$^2H_7$]-L-arginine in the sample of 10 μM and 50 μM, respectively.

Based on the explorative experiments the following experimental conditions were used to determine DDAH activity and perform validation experiments. Tissue samples (20 mg frozen tissue per determination of DDAH activity from a single organ) were homogenized in PBS buffer with protease inhibitor as described above to minimize possible interference by endogenously formed ADMA. The homogenate was centrifuged in a pre-cooled (4 °C) centrifuge for 5 min at 12,000 × *g*. For the DDAH activity assay 50 μl aliquots of the resulting supernatant were added to 50 μl aliquots of PBS buffer containing 20 μM [$^2H_6$]-ADMA and incubated for 60 min at 37 °C (final dilution (w/v) of the homogenate: 1:6 for kidney and 1:3 for liver). Reactions were stopped and [$^2H_6$]-ADMA was determined as described above. Enzyme activity was calculated as follows using an incubation time of 60 min: ([$^2H_6$]-ADMA in μmol/g protein at baseline *minus* [$^2H_6$]-ADMA in μmol/g protein after 60 min)/60 min.

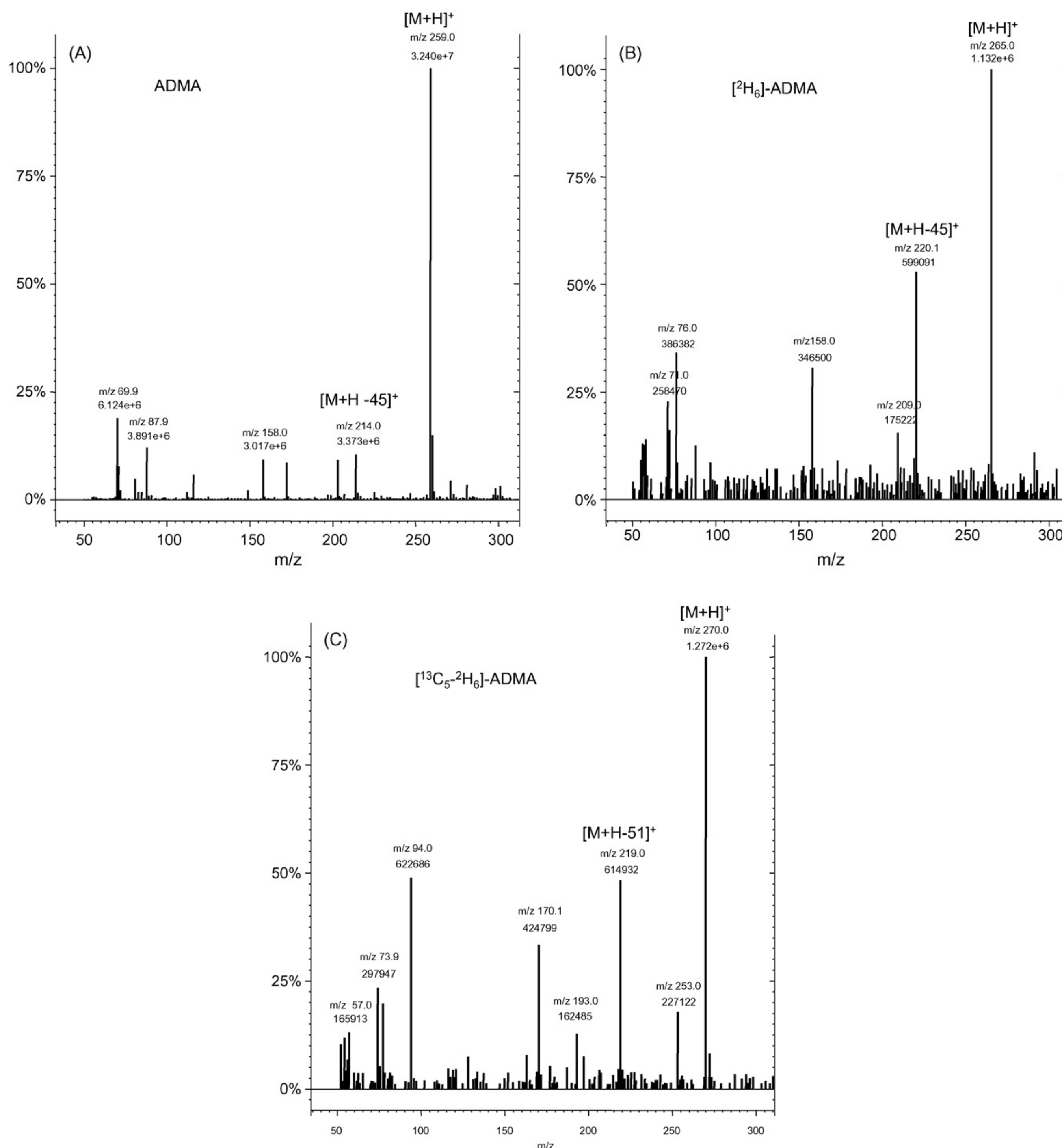

Fig. 3. Mass spectra of the butyl ester derivatives of ADMA (A), [3,3,4,4,5,5-$^2H_6$]-ADMA (B), and [1,1,1,1′,1′,1′-$^2H_6$-dimethylamino-1,2,3,4,5-$^{13}C_5$]-arginine ([$^{13}C_5$-$^2H_6$]-ADMA]) (C).

2.4. *Preparation of standards and samples*

[$^{13}C_5$-$^2H_6$]-ADMA and L-[$^2H_7$]-arginine were dissolved in methanol at concentrations of 1 μM and 5 μM, respectively. [$^{13}C_5$-$^2H_6$]-ADMA was used as internal standard for [$^2H_6$]-ADMA, ADMA and SDMA, L-[$^2H_7$]-arginine was used as internal standard for [$^{15}N_2$]-L-arginine and L-arginine. Before sample preparation the 96-well microfiltration plate had to be placed on top of a u-shaped 96-well polypropylene plate. Ninety six-well microfiltration plates were pre-loaded with 100 μl aliquots of the internal standard per well. Subsequently, to each well 10 μl of sample was added. Proteins were precipitated quantitatively by shaking the microfiltration plates on top of the u-shaped or multiply-PCR polypropylene plates for 5 min using an orbital shaker (Heidolph, Schwabach, Germany). To separate analytes from precipitated proteins, the microfiltration plates on top of the polypropylene plates were centrifuged for 5 min at 4000 rpm (Eppendorf, Hamburg, Germany). After

centrifugation, the two plates were separated and the eluates were dried by heating at 85 °C for 10 min. Compounds were derivatized, reconstituted, and analysed as described below. The calibrators were treated exactly the same as DDAH assay samples.

2.5. Derivatization and determination of ADMA, SDMA and L-arginine by LC–tandem MS

Quantification of unlabeled and stable-isotope labeled L-arginine, ADMA and SDMA was performed by LC–tandem MS by a modification of a previously described method [20,21]. All compounds were analysed as their butyl ester derivatives. Derivatization was performed in 96-well u-shaped 96-well polypropylene plates. After addition of 100 μl of 1 M HCl in 1-butanol, plates were sealed with aluminum foil (Sarstedt, Nuembrecht, Germany) and u-shaped 96-well polypropylene plates were heated at 65 °C on a 96-well aluminum block (Novodirect, Kehl, Germany) for 30 min. For evaporation, aluminum seal was removed and open plates were heated at 85 °C for 30 min. After evaporation of the derivatization reagent, samples were reconstituted in 100 μl aliquots of methanol–water, 50:50 (v/v) containing 0.1 wt.% ammonium formate, pH 4. The pH was adjusted with formic acid. Afterwards, polypropylene plates were transferred to an autosampler (HCS CombiPAL, CTC Analytics, Switzerland), and 10 μl aliquots were injected onto the chromatographic column for each sample.

LC–tandem MS analyses were performed on a Varian (Palo Alto, CA, USA) 1200L Triple Quadrupole MS equipped with two Varian ProStar model 210 HPLC pumps. Separation of analytes from major matrix components was achieved with a Chirobiotic T (20 mm × 1.0 mm i.d.) microbore guard column packed with teicoplanin covalently bonded to 5 μm particle size spherical silica (Astec, Whippany, NJ, USA). Acetonitrile containing 0.1 wt.% ammonium formate–water containing 0.1 wt.% ammonium formate, pH 4 (60:40, v/v), served as isocratic eluent at 28 °C, with a flow rate of 0.2 ml/min. Nitrogen was used as the nebulizing and drying gas (380 °C) at 90 and 180 l/h, respectively. For ionisation in the positive electrospray ionisation (ESI+) mode the needle and shield voltage were set at 5600 and 400 V, respectively.

3. Results

3.1. LC–MS and LC–tandem MS of L-arginine and dimethylated L-arginine butyl ester derivatives

The butylester derivatives of ADMA and SDMA showed a unique fragmentation pattern. ADMA and SDMA exhibit the same protonated molecular ion $[M+H]^+$, *m/z* 259, but only in the spectrum of ADMA the fragment $[M+H-45]^+$, *m/z* 214, is shown (Fig. 3A), while in the mass spectrum of SDMA this fragment is absent (not shown). $[M+H-45]^+$ was also observed for [3,3,4,4,5,5-$^2H_6$]-ADMA (*m/z* 220, Fig. 3B). For ADMA labeled with six deuterium atoms at the methyl moieties of the dimethylamino group, i.e. [1,1,1,1′,1′,1′-$^2H_6$]-[dimethylamino-1,2,3,4,5-$^{13}C_5$]-arginine, the same ion shows *m/z* 219, indicating that the stable-isotope labeled dimethylamine moiety is cleaved from the molecular ion, i.e. $[M+H-51]^+$ (Fig. 3C).

After fragmentation with argon (2 Pa) the following transitions were observed and used in quantitative analyses: *m/z* 231 to 70 [collision energy (CE) −22 eV] for L-arginine; *m/z* 238 to 77 [CE −22 eV] for L-[$^2H_7$]-arginine; *m/z* 259 to 214 [CE −16 eV] for ADMA; *m/z* 259 to 228 [CE −14 eV] for SDMA; *m/z* 265 to 220 [CE −16 eV] for [$^2H_6$]-ADMA, and *m/z* 270 to 219 for [$^{13}C_5$-$^2H_6$]-ADMA [CE −15 eV]. Total cycle time was 1 s. Representative partial chromatograms for the simultaneous multiple reaction monitoring (MRM) of L-arginine, L-[$^2H_7$]-arginine, ADMA, [$^2H_6$]-ADMA, [$^{13}C_5$-$^2H_6$]-ADMA, and SDMA are depicted in Fig. 4. The intra-assay coefficient of variation for the determination of endogenous ADMA and [$^2H_6$]-ADMA in matrix samples, i.e. kidney and liver homogenates diluted (w/v) 1:6 and 1:3, respectively, was 2.4% and 4.8%, respectively (Table 1). Inter-assay coefficients of variation are reported with the assay validation below.

3.2. Endogenous formation and metabolism of ADMA, SDMA and L-arginine

To establish optimal assay conditions we first explored endogenous turnover of ADMA, SDMA and L-arginine in the absence of protease inhibitors. In homogenates of renal tissue of C57BL6 mice ($n=5$) prepared separately and incubated in parallel for 1 h at 37 °C there was a net formation of L-arginine of 5160 ± 690 nmol/g protein, ADMA and SDMA content increased by 114 ± 12 nmol/g protein and 36 ± 4 nmol/g protein, respectively. This corresponded to a relative increase of L-arginine, ADMA and SDMA by 200–400% as compared to baseline (Fig. 5). Endogenous formation of ADMA was inhibited by a protease inhibitor mix (Fig. 6).

DDAH activity was assessed by addition of [$^2H_6$]-ADMA (final concentration 3 μM) to mouse kidney homogenates (Fig. 7). Using otherwise identical assay conditions as before we observed after incubation for 1 h a net degradation of [$^2H_6$]-ADMA by 59 ± 2.4 nmol/g protein. Addition of 500 μM of L-NMMA, which acts as a competitive inhibitor of the ADMA-metabolizing enzyme DDAH [9], significantly reduced degradation of [$^2H_6$]-ADMA to 29 ± 5.4 nmol/g protein ($p<0.01$). At the same time net generation of endogenous ADMA increased from 119 ± 23 nmol/g protein to 207 ± 15 μmol/g protein after addition of 500 μM of L-NMMA ($p=0.01$), indicating impaired degradation of endogenously formed ADMA by DDAH.

3.3. Validation of the DDAH activity assay

Based on the previous explorative experiments we established optimal assay conditions for small frozen tissue samples as detailed in Section 2. For these conditions we studied the inter-assay precision (RSD) for the enzyme activity, which was determined to be 8.9% in kidney and 10% in liver tissue homogenates (Table 1).

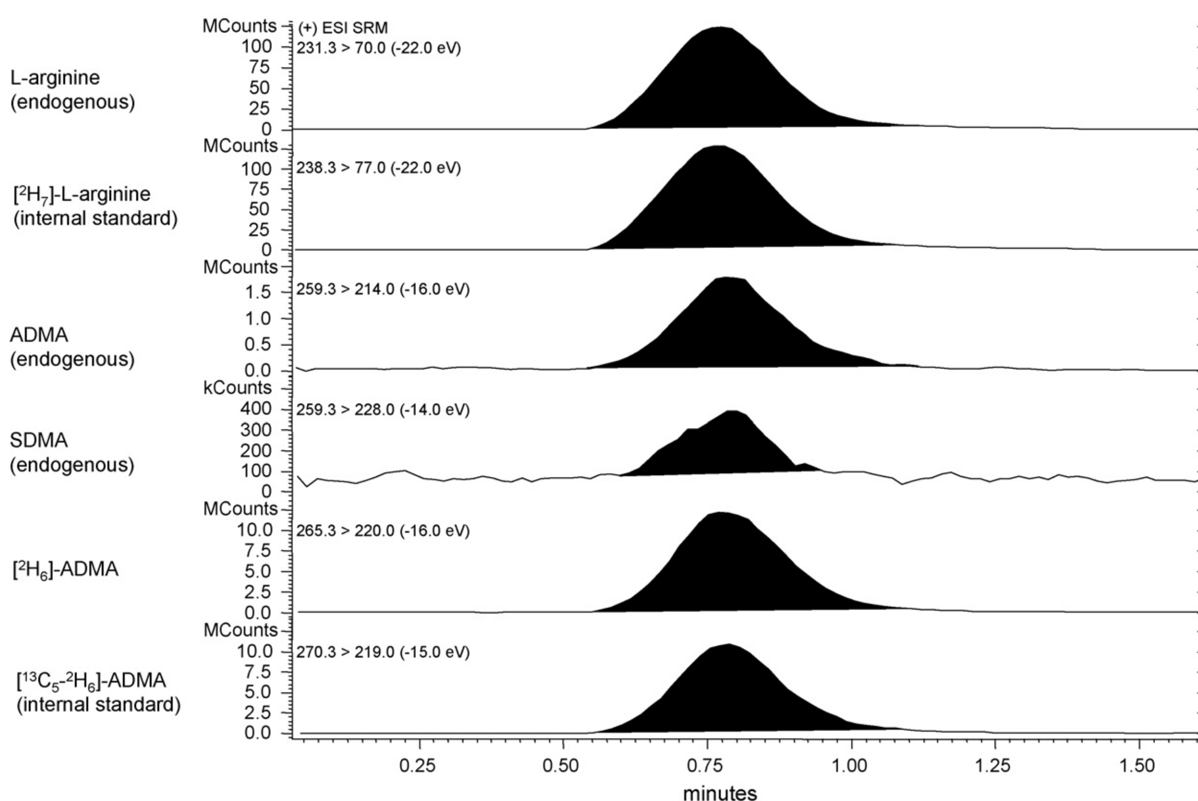

Fig. 4. Partial chromatograms from the simultaneous LC–tandem MS analysis of the butyl ester derivatives of L-arginine, L-[$^2H_7$]-arginine (internal standard), SDMA (endogenous), ADMA (endogenous); [$^2H_6$]-ADMA added to achieve a final concentration of 10 μM; [$^{13}C_5$-$^2H_6$]-ADMA (internal standard) in homogenates of mouse kidney.

4. Discussion

The present stable-isotope based assay permits reliable and reproducible determination of DDAH activity in small tissue samples. Furthermore, in our explorative experiments using stable-isotope labeled ADMA and L-arginine analogues we were able to simultaneously assess endogenous formation and metabolism of ADMA, SDMA and L-arginine in a single run, which has not been possible so far.

To allow first practical application of the stable-isotope based assay we optimized and validated assay conditions for mouse kidney and liver, based on protein weight-corrected data obtained after a fixed incubation time of 60 min. These organs were chosen as they are known to be significantly involved in ADMA metabolism and thus, constitute promising targets for therapeutic interventions [4]. The possibility to perform the DDAH activity assay using small (20 mg) tissue samples in a 96-well format and the short LC–tandem MS run times should facilitate application of the assay in preclinical research and allows to easily upscale the number of samples processed.

Our data obtained from method validation indicate that at least in kidney tissue of the mouse there is considerable endogenous formation of ADMA, SDMA and L-arginine. This observation is in line with recent data obtained by determination of unlabeled ADMA by HPLC in whole blood preparations [11] and rat kidney [14]. Our data also pinpoint several possible pitfalls associated with most presently used methods to assess DDAH activity and indicate that stable-isotope techniques may help to overcome some of these limitations. Presently, when using unlabeled ADMA as a substrate only the net effect of ADMA degradation or L-citrulline generation can be assessed, the use of stable-isotope labeled ADMA allows to overcome

Table 1
Inter-assay precision (RSD, %) of the DDAH activity assay in kidney and liver homogenates

Tissue	[$^2H_6$]-ADMA content at baseline (nmol/g protein)	Precision (RSD, %)	[$^2H_6$]-ADMA content after 1 h (nmol/g protein)	Precision (RSD, %)	DDAH activity (nmol [$^2H_6$]-ADMA/g protein × h)	Precision (RSD, %)
Kidney	383 ± 13	3.4	193 ± 13	6.7	191 ± 17	8.9
Liver	131 ± 8	6.1	60 ± 2	3.3	70 ± 7	10.0

To assay DDAH activity, homogenates of mouse kidney (n = 6) and liver (n = 4) were prepared from 20 mg tissue samples, diluted (kidney 1:6, liver 1:3, w/v) in PBS buffer with protease inhibitor and incubated for 60 min at 37 °C with [$^2H_6$]-ADMA at a final concentration of 10 μM. PBS contained NaCl 137 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM, and had a pH value of 6.4 adjusted by HCl. Protease inhibitor mix contained aprotinin 0.08 mM, AEBSF 104 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM diluted 1:20, v/v).

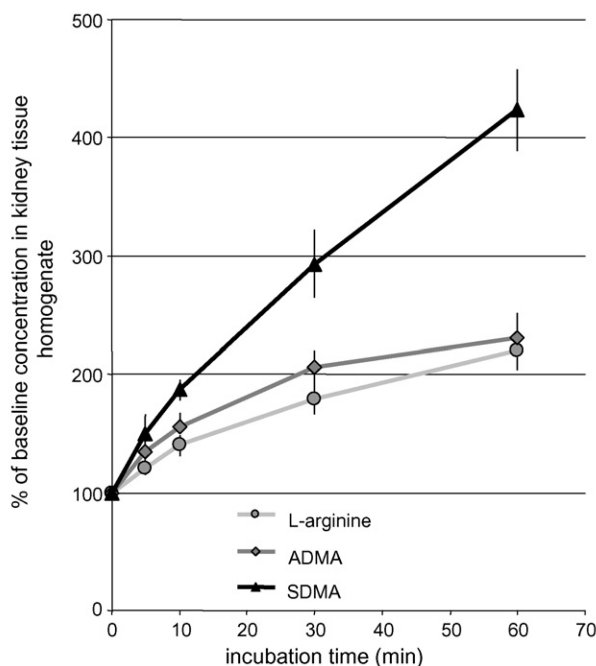

Fig. 5. Endogenous formation of ADMA, SDMA and L-arginine assessed in mouse kidney homogenates (1:4, w/v, in PBS buffer, pH 6.4) of five different animals in the absence of protease inhibitor for 1 h at 37 °C. Mean baseline content of ADMA, SDMA and L-arginine was 77 ± 14, 10 ± 1, and 4380 ± 175 nmol/g protein, respectively.

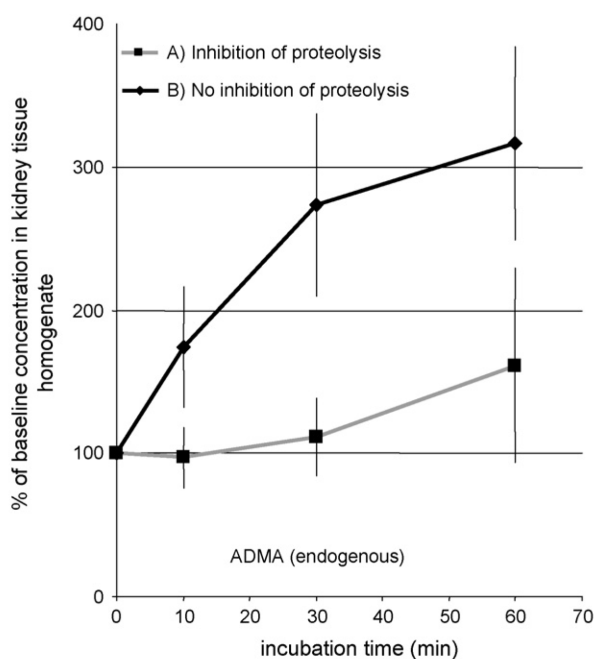

Fig. 6. Inhibition of endogenous formation of ADMA in kidney tissue homogenates of five different mice (diluted 1:4, w/v, in PBS buffer, incubated at 37 °C for 1 h) in presence (A) or absence (B) of a protease inhibitor mix standard (aprotinin 0.08 mM, AEBSF 104 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin A 1.5 mM, E-64 1.4 mM in DMSO, Sigma, Germany) added at a final dilution of 1:20, v/v. Baseline content of ADMA was 38 ± 18 and 47 ± 12 nmol/g protein in absence and presence of a protease inhibitor mix, respectively.

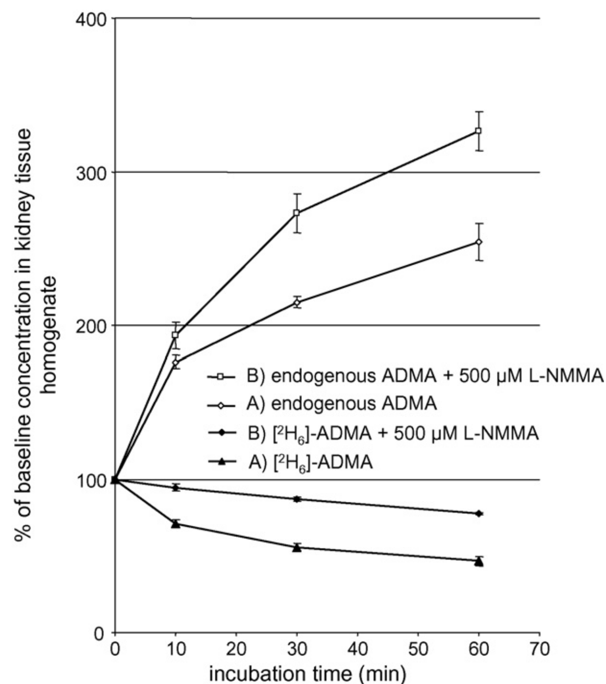

Fig. 7. Endogenous formation of ADMA and degradation of $[^2H_6]$-ADMA (3 μM) assessed in mouse kidney homogenates (1:5, w/v, in PBS buffer, pH 6.4) of five different animals incubated for 1 h at 37 °C in the presence (A) and absence (B) of 500 μM of L-NMMA (to competitively inhibit DDAH activity). Mean ± SEM baseline content (=100%) of endogenous ADMA was 67 ± 1 nmol/g protein for condition (A) and 91 ± 1 nmol/g for condition (B), whereas baseline content (=100%) of $[^2H_6]$-ADMA was 105 ± 1 nmol/g protein for condition (A) and 122 ± 1 nmol/g protein for condition (B).

this limitation, as in vivo and in vitro there is no formation of stable-isotope labeled ADMA.

Formation of ADMA has previously been attributed to endogenous protein turnover and proteolysis [11,14], and indeed we have demonstrated in the present study that it could be attenuated by protease inhibition. Moreover, we could demonstrate that formation of ADMA is further increased by competitive inhibition of DDAH by L-NMMA. Especially in tissue samples and in vivo, where the plasma and tissue ADMA concentration represents a steady state of ADMA formation and metabolism, this observation may have very practical implications when assessing drug effects on activity of DDAH or protein–arginine–methyltransferases (PRMT), the enzymes responsible for the formation of methylated L-arginine in proteins [4]. The simplest assays for DDAH activity rely on unlabeled ADMA as a substrate and simply measure the remaining ADMA concentration after a given incubation period [11,12]. This may be perfect when studying the purified DDAH enzyme but interpretation of results can become very difficult when using unlabeled ADMA in vivo, in tissues or in cell culture. Here the ADMA concentration at any given time point rather represents a steady state of protein methylation and subsequent liberation of ADMA by proteolysis, hydrolysis by DDAH, cellular transport and excretion in vivo measurements.

In tissues with high DDAH activity this limitation can partly be overcome by adding unlabeled ADMA in vast excess of the endogenous concentrations or by extensive dilution of the

sample, and thus limiting the protein content and the possible endogenous formation of ADMA. DDAH appears to require no cofactors and also to be a highly active enzyme, thus, a considerable dilution of tissue homogenates should be possible [9,22]. Where dilution is not feasible, addition of protease inhibitors which apparently do not significantly affect DDAH activity may provide an alternative. It has to be kept in mind, however, that protease inhibitors are quite toxic and may not be suitable for in vivo, cell culture or organ studies.

At very high concentrations ADMA may also impair L-arginine transport via the y^+ transporters [23] and thus, seriously affect cell growth and L-arginine-dependent physiology. The alternative approach of assessing formation of L-citrulline from endogenous ADMA has its limitations, too, as several alternative pathways contribute to the formation of L-citrulline (Fig. 1). Thus, in tissue rich in arginase activity, such as in the liver or in whole blood, endogenous L-arginine is rapidly metabolized to L-ornithine, which in turn is converted to L-citrulline. Moreover, due to their structural similarity to ADMA, L-citrulline and L-arginine may directly impair DDAH activity [9].

In case added ADMA concentrations in a DDAH activity assay are below the IC_{50} of the NOS enzymes residual or induced NOS activity in several tissues such as vascular, neuronal or inflamed tissues will generate not only NO but also L-citrulline. In both cases stable-isotope labeled L-arginine could be used to assess L-arginine metabolism in order to get a better estimate of the relative contribution of these pathways.

So far, DDAH activity assays based on the metabolism of radio-labeled ADMA, i.e. ^{14}C-ADMA [9], or ^{14}C-L-NMMA [10] are considered the “gold standard”. Using ^{14}C-ADMA, non-radioactive ADMA formed endogenously during the assay is not detected simultaneously and thus, does not directly lead to false low estimates of DDAH activity. This leaves some caveats with these methods, however. Unlabeled ADMA may not be detected by such radio-labeled based assays and the endogenously formed ADMA can competitively inhibit degradation of ^{14}C-ADMA by DDAH. A drug inhibiting proteolysis or protein methylation would reduce endogenous formation of ADMA and thus, reduce competitive inhibition of DDAH, possibly leading to the wrong conclusion that the drug may directly augment DDAH activity. The stable-isotope technique can overcome these limitations as formation and metabolism of ADMA can be measured simultaneously.

In certain settings, such as in vivo studies in humans and large animals, the use of protease inhibitors may not be feasible and the use of stable-isotope labeled ADMA may be too expensive. Here, simultaneous determination of ADMA and SDMA may allow obtain a rough estimate of the extent of endogenous ADMA and L-arginine generation. Like ADMA and L-arginine, SDMA is also formed when proteins are degraded but it appears not to be actively metabolized, thus the rate of SDMA accumulation may provide an estimate of protein turnover and ADMA formation.

As a further alternative, assessment of DDAH activity could be based on the formation of the ADMA metabolite dimethylamine (DMA) or on changes in the molar ratio of DMA and ADMA [24]. However, ADMA is not the only source of DMA, and especially in vivo several alternative sources of DMA including diet have to be considered [24,25]. Also, ADMA and DMA have to be determined separately [24], so far. In principle, the use of stable-isotope labeled ADMA would permit to overcome some of these limitations. Estimates of DDAH activity could be based on the formation of the stable-isotope labeled ADMA metabolites L-citrulline or DMA or changes in the molar ratio of DMA and ADMA.

The present study was designed to prove the principle that formation and metabolism of ADMA and related compounds can be measured in parallel by LC–tandem MS. Therefore, not all factors possibly interfering with DDAH activity were assessed at the present stage. Cellular transport was also not assessed in the present study, but application of the stable-isotope LC–tandem MS technique is certainly feasible and will most likely add further complexity to present perceptions of L-arginine and ADMA metabolism.

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